Abl Family Kinases Regulate Endothelial Function

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

The vasculature has a crucial function in normal physiology, enabling the transport of oxygen and nutrients to cells throughout the body. In turn, endothelial cells, which form the inner-most lining of blood vessels, are key regulators of vascular function. In addition to forming a barrier which separates the circulation from underlying tissues, endothelial cells respond to diverse extracellular cues and produce a variety of biologically-active mediators in order to maintain vascular homeostasis. Disruption of normal vascular function is a prominent feature of a variety of pathological conditions. Thus, elucidating the signaling pathways regulating endothelial function is critical for understanding the role of endothelial cells in both normal physiology and pathology, as well as for potential development of therapeutic interventions.

In this dissertation, we use a combination of pharmacological inhibition and knockdown studies, along with generation of endothelial conditional knockout mice, to demonstrate an important role of the Abelson (Abl) family of non-receptor tyrosine kinases (Abl and Arg) in vascular function. Specifically, loss of endothelial expression of the Abl kinases leads to late-stage embryonic and perinatal lethality in conditional knockout mice, indicating a crucial requirement for Abl/Arg kinases in normal vascular development and function. Endothelial *Abl*/*Arg*-null embryos display focal regions of

vascular loss and tissue damage, as well as increased endothelial cell apoptosis. An important pro-survival function for the Abl kinases is further supported by our finding that either microRNA-mediated *Abl*/*Arg* depletion or pharmacological inhibition of the Abl kinases increases endothelial cell susceptibility to stress-induced apoptosis *in vitro*. The Abl kinases are activated in response to treatment with the pro-angiogenic growth factors vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). We show that both VEGF- and bFGF-mediated endothelial cell survival is impaired following Abl kinase inhibition.

These studies have uncovered a previously unappreciated role for the Abl kinases in the regulation of the angiopoietin/Tie2 signaling pathway, which functions to support endothelial cell survival and vascular stability. Loss of Abl/Arg expression leads to reduced mRNA and protein levels of the Tie2 receptor, resulting in impaired activation of intracellular signaling pathways by the Tie2 ligand angiopoietin-1 (Angpt1), as well as decreased Angpt1-mediated endothelial cell survival following serum-deprivation stress. Notably, we found that the Abl kinases are activated following Angpt1 stimulation, suggesting a unique dual role for Abl and Arg in Angpt/Tie2 signaling, potentially modulating Tie2 downstream signaling responses, as well as regulating Tie2 receptor expression.

Further, we show an important contribution of the Abl family kinases to the regulation of endothelial permeability responses both *in vitro* and *in vivo*. The Abl

kinases are activated in response to a diverse group of permeability-inducing factors, including VEGF and the inflammatory mediators thrombin and histamine. We show that inhibition of Abl kinase activity, using either the ATP-competitive inhibitor imatinib or the allosteric inhibitor GNF-2, protects against disruption of endothelial barrier function by the permeability-inducing factors *in vitro*. VEGF-induced vascular permeability similarly is decreased in conditional knockout mice lacking endothelial *Abl* expression, as well as following treatment with Abl kinase inhibitors *in vivo*. Mechanistically, we show that loss of Abl kinase activity is accompanied by activation of the barrier-stabilizing GTPases (guanosine triphosphatases) Rac1 and Rap1, as well as inhibition of agonist-induced Ca^{2+} mobilization and generation of acto-myosin contractility.

Taken together, these results demonstrate involvement of the Abl family kinases in the regulation of endothelial cell responses to a broad range of pro-angiogenic and permeability-inducing factors, as well as a critical requirement for the endothelial Abl kinases in normal vascular development and function *in vivo*. These findings have implications for the clinical use of Abl kinase inhibitors.

Dedication

To my family, who have always had faith in me, even at times when I've lost it in myself. Thank you for your patience, love, and support. I love you all so much. -Beth

Contents

List of Tables

List of Figures

List of Abbreviations

Abi	Abl-interactor
Abl	Abelson kinase
Abl^{ECKO}	endothelial *Abl* knockout
ALI	acute lung injury
AMD	age-related macular degeneration
Angpt	angiopoietin
Angpt1	angiopoietin-1
Arg	Abl-related gene
Arp2/3	actin-related protein 2/3
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BCR-ABL1	breakpoint cluster region-Abl fusion oncoprotein
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
C-lobe	C-terminal lobe
CML	chronic myelogenous leukemia

DAG	diacylglycerol
DEP-1	density-enhanced phosphatase-1
DNA	deoxyribonucleic acid
Dok-R	downstream of tyrosine kinase-related protein
E	embryonic day
EBM-2	endothelial basal medium-2
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
Erk	extracellular signal-regulated kinase
F	phenylalanine
F-actin	filamentous actin
FAK	focal adhesion kinase
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FOXO1	Forkhead box protein O1
GAP	GTPase-activating protein

GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
Grb	growth factor receptor-bound protein
GTPase	guanosine triphosphatase
H&E	hematoxylin/eosin
HBSS	Hank's Balanced Salt Solution
HEK	human embryonic kidney
HIF	hypoxia-inducible factor
HMVEC	human microvascular endothelial cell
HUVEC	human umbilical vein endothelial cell
I	isoleucine
IAP	inhibitor of apoptosis
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobulin
IL	interleukin
IP_3	inositol-1,4,5-trisphosphate
K	lysine
KLF2	Kruppel-like factor 2
LLC	Lewis Lung Carcinoma
LPS	lipopolysaccharide

MAPK	mitogen-activated protein kinase
mAbl (mArg)	murine Abl (Arg)
mRNA	messenger RNA
MEF	mouse embryo fibroblast
MEK	mitogen-activated protein kinase kinase
miRNA	microRNA
MLC2	myosin regulatory light chain
MLCK	myosin light chain kinase
MT1-MMP	membrane type 1 matrix metalloproteinase
N-cadherin	neural cadherin
NO	nitric oxide
PAK	p21-activated kinase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFR	PDGF receptor
PECAM-1	platelet endothelial cell adhesion molecule
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C

PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PTP-PEST	PEST-type protein tyrosine phosphatase
PyMT	Polyoma middle T antigen
R	arginine
Rb	retinoblastoma protein
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
ROCK	Rho kinase
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription-PCR
S	serine
S1P	sphingosine-1-phosphate
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SH	Src homology
Shp2	SH2-containing protein tyrosine phosphatase
T	threonine

TBS-T	Tris-buffered saline-Tween 20
TCR	T-cell receptor
TNFα	tumor necrosis factor α
UT	untreated; unstimulated; vehicle-treated
VCAM-1	vascular cell adhesion molecule-1
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
vWF	von Willebrand factor
WASP	Wiscott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein
WGA	wheat germ agglutinin
WT	wild-type
Tie2	tyrosine kinase with Ig-like and EGF homology domains
Y	tyrosine

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1. Background and Significance

The vasculature plays an important role in normal physiology, enabling the transport of oxygen and nutrients to cells throughout the body. The endothelium, which forms the inner-most lining of blood vessels, functions to separate the circulation from the underlying tissues. While early vascular biologists thought of endothelial cells as forming a static barrier, "a sheet of nucleated cellophane [1]," it has become clear that the endothelium is actually a dynamic, multifunctional organ. Endothelial cells are key players in vascular function, responding to diverse extracellular cues, as well as producing a variety of biologically-active mediators, in order to maintain vascular homeostasis [2]. In addition to forming a semi-permeable barrier between tissues and the bloodstream, which controls the transport of solutes and immune cells into and out of the circulation, endothelial cells regulate vasoreactivity and act to maintain the vasculature in an anti-thrombotic state [2]. As disruption of vascular homeostasis is a key feature of a variety of pathological conditions [2,3,4], elucidating the signaling pathways regulating endothelial function is critical for understanding the role of endothelial cells in both normal physiology and pathology, as well as for potential development of therapeutic interventions.

1.1 Vascular Development

The mature vasculature exists as an extensive network of interconnected, hierarchically-organized blood vessels. This complex vascular network is generated

primarily through two broad mechanisms, vasculogenesis and angiogenesis. Vasculogenesis is the process whereby new blood vessels are created *de novo* following endothelial cell differentiation from multipotent progenitor cells, while angiogenesis involves expansion of the vascular network through sprouting or splitting of existing blood vessels [5].

1.1.1 Vasculogenesis

The cardiovascular system is the first organ system to form during vertebrate embryonic development [6]. The initial blood vessels are formed through the *in situ* differentiation of mesodermal precursors (angioblasts) into endothelial cells. The process of vasculogenesis occurs between embryonic day (E) 6 and E10 in mice [7] and is initially observed in the extra-embryonic yolk sac membrane, where mesodermal precursors cells aggregate to form blood islands. Cells on the periphery of these blood islands (angioblasts) differentiate into endothelial cells, while the inner cells (hematopoietic progenitor cells) differentiate into blood cells. Within the embryo, angioblasts similarly differentiate to form intra-embryonic endothelial cells. These nascent endothelial cells coalesce to form vascular tubes, which anastamose to form a primitive vascular network, known as the primary capillary plexus [6,8], a process involving alterations in endothelial cell morphology, as well as modulation of cell-cell and cell-matrix adhesion [9]. Vascular endothelial growth factor (VEGF) is a crucial regulator of vasculogenesis. Indeed, mouse embryos lacking expression of VEGF receptor 2 (VEGFR2; also known as

Flk1 in mice or *Kdr* in humans) die between E8.5 and E9.5; these embryos lack blood islands and organized blood vessels and exhibit diminished populations of hematopoietic progenitors [10].

1.1.2 Angiogenesis

The initial unorganized vascular network undergoes a program of expansion, pruning, and remodeling in order to form a mature, functional vasculature [9]. Growth of the vasculature occurs through the process of angiogenesis, whereby new blood vessels are formed from pre-existing vessels. Angiogenic growth can occur either through sprouting mechanisms, in which new vessels form by branching off from existing vascular channels, or through non-sprouting mechanisms involving splitting of existing vessels (i.e., intussusception) [9]. The mechanisms regulating sprouting angiogenesis have been most well-characterized.

Sprouting angiogenesis is a complex process, involving the coordinated regulation of a variety of endothelial cell processes, including migration, proliferation, survival, and capillary morphogenesis [11]. Initiation of angiogenic sprouting requires loosening of endothelial intercellular junctions, enabling endothelial migration in the direction of an angiogenic stimulus [5]. The growing vascular sprouts are each lead by a specialized endothelial tip cell that dynamically extends numerous filopodia, which enable the tip cell to respond to a variety of attractant and repellant stimuli to guide the extension of the nascent sprout [8]. While the leading endothelial tip cells mediate

directional migration of vascular sprouts, the trailing endothelial cells, known as stalk cells, proliferate to extend the stalk of the new vessel [12]. Endothelial tip cells also express the metalloproteinase MT1-MMP, which mediates degradation of the surrounding extracellular matrix (ECM), allowing for endothelial cell invasion and migration during vascular sprouting [13].

In addition to its role in vasculogenesis, VEGF is a particularly important regulator of sprouting angiogenesis. VEGF-A (VEGF), the primary pro-angiogenic member of the VEGF family of ligands, signals through the receptor tyrosine kinase VEGFR2 to promote endothelial cell proliferation, migration, and survival [14]. VEGFR2 is highly expressed in endothelial tip cells, particularly in their filopodia [12]. VEGF stimulates extension of tip cell filopodia to promote directional migration and promotes proliferation of stalk cells. VEGF signaling is also a critical determinant of tip cell identity, through the modulation of Notch pathway activation [15]. VEGFR2 activation in tip cells leads to upregulation of the Notch ligand Dll4, which in turn activates Notch signaling in neighboring stalk cells, leading to downregulation of VEGFR2 and upregulation of the related VEGFR1. As a result, stalk cells become less responsive to VEGF, compared to the leading tip cells. Interestingly, tip cell selection is a dynamic process, influenced by relative levels of VEGF and Notch pathway activation, which allows for the continual modulation of angiogenic sprouting in response to extracellular cues [16].

1.1.3 Vascular Remodeling and Maturation

Following angiogenic expansion, remodeling of the vascular network, accompanied by selective pruning (regression) of excess vascular branches, results in the generation of a highly-organized, hierarchically-branched vascular system, with tissue-specific vascular patterning and vessel density [17]. The establishment of blood flow, along with the activity of ephrin and Notch signaling pathways, contributes to the remodeling of the vascular network to form arteries, veins, and capillaries [8]. However, differential expression of arterial (ephrin-B2) and venous (EphB4 receptor) markers has been detected in the developing endothelium even prior to onset of circulation, suggesting genetic regulation of arterial versus venous specification [18]. Following the establishment of circulation, blood is pumped from the heart through the arterial system to tissue capillary beds, where exchange of gases and nutrients occur, prior to return of the blood to the heart via the venous circulation [8]. Interstitial fluid, which results from plasma leakage from the capillaries into the surrounding tissues, is returned to the venous circulation through the lymphatic system [19]. The lymphatic vasculature, which has an important role in the maintenance of fluid balance, is formed by sprouting from embryonic veins, regulated by signaling mediated by the VEGF family member VEGF-C.

Formation of a mature, functional vasculature also requires recruitment of mural cells, which surround the endothelial tubes and function to stabilize the vessels [20].

Larger vessels are surrounded by multiple layers of contractile vascular smooth muscle cells, while smaller vessels are covered by varying numbers of pericytes. These periendothelial cells stimulate ECM production to stabilize nascent vessels, as well as inhibit endothelial cell proliferation and migration [5]. Mural cells also support endothelial cell survival, in part through the production of soluble mediators including angiopoietin-1 (Angpt1) [21]. The critical supportive function of these periendothelial cells is illustrated by the excessive vessel regression observed following disruption of endothelial cell-pericyte interactions in the retinal vasculature [22]. Platelet-derived growth factor (PDGF) signaling is an important mediator of vascular maturation. Endothelial cells secrete PDGF-B, which binds to the PDGF receptor β (PDGFRβ) on pericytes and vascular smooth muscle cells, promoting mural cell recruitment [21]. Genetic ablation of PDGF-B or PDGFRβ in mice impairs mural cell coverage of blood vessels [23], contributing to vascular abnormalities including endothelial hyperplasia, vessel dilation, and impaired barrier function [24], as well as microaneurysms, hemorrhage and edema [25,26,27,28].

1.2 Endothelial Cell-Cell and Cell-Matrix Adhesion

In contrast to the angiogenic growth of the vasculature during embryonic development and postnatal growth, the endothelial cells of the adult vasculature are typically quiescent [29]. However, while quiescent, the mature endothelium is not simply a static barrier, but rather, an important regulator of vascular homeostasis [2].

Endothelial cell-cell and cell-matrix adhesions are critical for proper vascular function, acting to maintain vascular structure and modulate endothelial cell growth factor signaling responses. The major structural components of endothelial cell-cell and cell-matrix adhesions are shown in **Figure 1.1**.

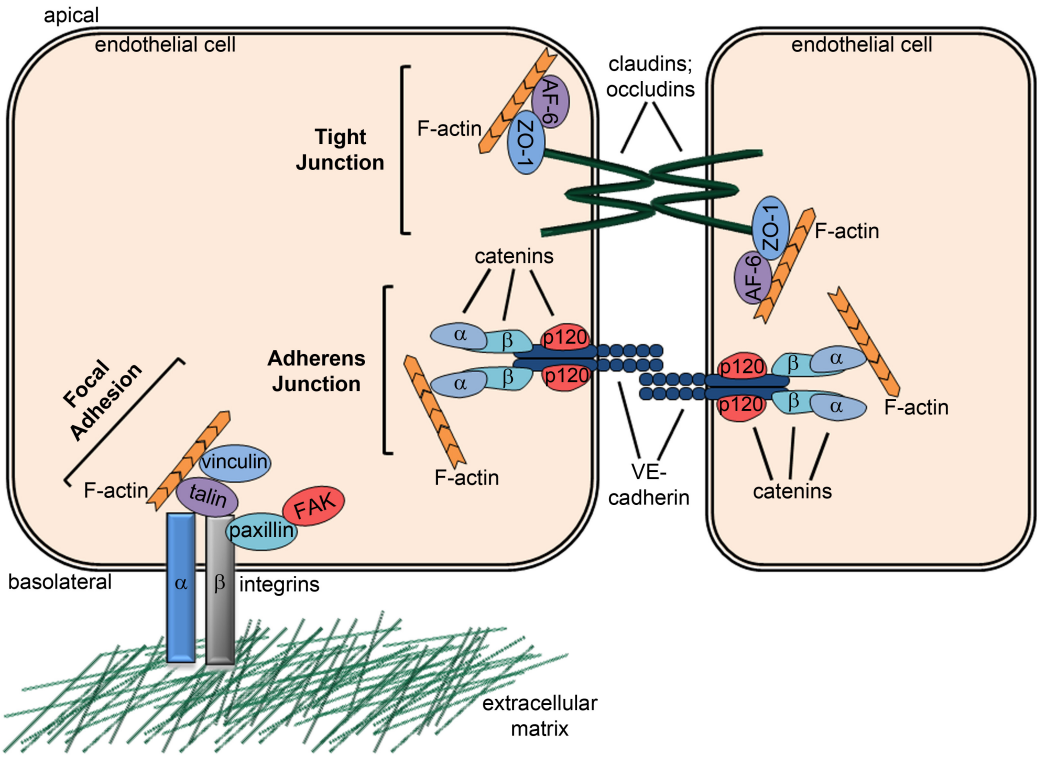

Figure 1.1 Structural Components of Endothelial Cell-Cell and Cell-Matrix Adhesions

Endothelial barrier function and structural integrity are maintained by cell-cell and cell-matrix adhesions. The two major types of endothelial cell-cell adhesions are formed by tight junction and adherens junction protein complexes. The primary structural components of tight junctions are transmembrane proteins of the claudin and occludin families, which are connected to the actin cytoskeleton through interaction with actin-binding proteins including ZO-1 and AF-6/afadin. Adherens junctions are composed of the transmembrane protein vascular endothelial (VE)-cadherin, which binds intracellularly to catenin proteins (β-catenin, α-catenin, and p120-catenin) that function to link VE-cadherin to the underlying actin cytoskeleton, as well as regulate VE-cadherin

internalization. Endothelial cell-matrix adhesions are mediated by the binding of integrin heterodimers (α,β) to the extracellular matrix. The short cytoplasmic tails of integrins interact with actin-binding proteins (including talin and vinculin), adaptor proteins (paxillin), and signaling mediators (focal adhesion kinase, FAK) to form focal adhesion complexes.

1.2.1 Intercellular Junctions

The endothelium forms a critical semi-permeable barrier between tissues and the bloodstream. Maintenance of this barrier is a dynamic and tightly-controlled process, which regulates the transport of solutes and immune cells into and out of the circulation. While loosening of the endothelial barrier is an important aspect of both normal angiogenic remodeling and inflammatory responses [5,30], abnormally elevated vascular permeability is a key feature of a variety of pathological conditions, contributing to edema, increased interstitial fluid pressure, and tissue damage [31,32]. Endothelial cells express a number of cell surface adhesion molecules that cluster at interendothelial cell-cell junctions to maintain vascular integrity. The two major types of intercellular junctions present in endothelial cells are tight junctions and adherens junctions, which together regulate permeability of the endothelial barrier [33]. Along with the adhesion proteins that form tight junctions and adherens junctions, endothelial cells express several other cell adhesion molecules which localize to interendothelial junctions, including platelet endothelial cell adhesion molecule (PECAM-1/CD31) [33]. PECAM-1, which also is expressed by monocytes and neutrophils, promotes

transendothelial migration of leukocytes [34], likely through homophilic binding between endothelial cell and leukocyte PECAM-1 proteins.

1.2.1.1 Tight Junctions

Tight junctions are formed primarily by integral membrane proteins of the claudin and occludin families. Junctional adhesion molecule (JAM) proteins, which are transmembrane proteins of the immunoglobulin superfamily, also are found in tight junction protein complexes, as is the related endothelial cell-selective adhesion molecule (ESAM) [35]. These proteins cluster in rows to form tight junction complexes, which associate with complexes on the membrane of the neighboring endothelial cell to seal the intercellular space [36]. Tight junction components associate intracellularly with a variety of PDZ domain-containing proteins, including the ZO proteins (ZO-1, ZO-2, and ZO-3), AF-6/afadin, and the polarity regulator PAR-3 [37]. ZO proteins and AF-6 bind actin filaments, thus linking the tight junction protein complexes to the cortical actin cytoskeleton. While most tight junction components are expressed in a variety of cell types, the claudin family member claudin-5 is expressed specifically in endothelial cells [38]. Mice lacking claudin-5 die perinatally as a result of size-selective loosening of the blood-brain barrier [39], demonstrating the importance of tight junctions in maintaining endothelial barrier integrity. Notably, the frequency of tight junctions varies considerably between different types of vessels or vascular beds. While tight junctions are abundant in the blood vessels of the central nervous system, forming the highly

impermeable blood-brain barrier, these junctions are less numerous in regions of the vasculature characterized by dynamic transport of solutes or leukocytes, such as the post-capillary venules [33].

1.2.1.2 Adherens Junctions

In contrast to tight junctions, adherens junctions, which are formed by transmembrane proteins of the cadherin family, are found more uniformly throughout the vasculature [40]. Endothelial cells express both vascular endothelial cadherin (VE-cadherin) and neural cadherin (N-cadherin) [41]. N-cadherin does not localize to endothelial cell-cell junctions [42], but instead is thought to mediate association of endothelial cells with surrounding pericytes [43]. In contrast, VE-cadherin is the major structural component of interendothelial adherens junctions and is a critical regulator of vascular integrity and endothelial barrier function [44]. Dimerization and clustering of VE-cadherin at sites of endothelial cell-cell contact leads to homotypic, Ca^{2+}-dependent interaction of the extracellular domains of VE-cadherin proteins on neighboring cells. The distal portion of the VE-cadherin intracellular domain binds to β-catenin or plakoglobin, which indirectly link VE-cadherin to the actin cytoskeleton through α-catenin binding [45]. α-catenin interacts with actin filaments, as well as actin-binding proteins including vinculin and α-actinin [33]. However, α-catenin appears not to interact simultaneously with VE-cadherin protein complexes and with actin filaments [46], suggesting dynamic linkage of adherens junctions with the underlying actin

cytoskeleton. In lymphatic vessels, plakoglobin can also bind to desmoplakin, which mediates association of VE-cadherin with the vimentin cytoskeleton at endothelial cell-specific desmosome-like structures known as complexus adherens [47]. The intracellular domain of VE-cadherin also contains a proximal binding site for p120-catenin; p120-catenin association functions to inhibit VE-cadherin endocytosis [48].

Genetic depletion of components of the adherens junction complex in mice has demonstrated the important role of these proteins in vascular development and vessel integrity. Endothelial *β-catenin* inactivation leads to embryonic lethality between E11.5 and E13.5, with mutant embryos displaying abnormal vascular patterning and fragile, hemorrhagic vessels [52]. Genetic depletion of *VE-cadherin*, or expression of a VE-cadherin cytosolic truncation mutant lacking the β-catenin binding-domain, led to embryonic death at E9.5, as a result of defective angiogenic remodeling and extensive vascular disintegration and regression [49]. Increased endothelial apoptosis was observed in *VE-cadherin*-deficient embryos, suggesting an important pro-survival function for VE-cadherin. Indeed, VE-cadherin was demonstrated to physically associate with VEGFR2, promoting VEGF-mediated activation of the pro-survival phosphoinositide 3-kinase (PI3K)/Akt pathway [49]. The VE-cadherin/VEGFR2 complex inhibits VEGF-induced activation of the p44/42 mitogen-activated protein kinase (MAPK, also known as extracellular signal-regulated kinase, Erk), leading to contact inhibition of VEGF-induced proliferation in confluent endothelial cells [50]. This

modulation of VEGF signaling occurs through VEGFR2 dephosphorylation mediated by the VE-cadherin-associated tyrosine phosphatase density-enhanced phosphatase-1 (DEP-1), leading to inhibition of VEGFR2 internalization and proliferative signaling [50,51].

1.2.2 Cell-Matrix Adhesions

On the basolateral surface, endothelial cells are separated from the underlying interstitium by a thin ECM layer known as the basal lamina [52]. Endothelial cells synthesize and secrete a variety of ECM proteins, including collagen IV, fibronectin, and laminin [53]. Endothelial cells interact with the surrounding ECM through integrins, which are heterodimeric adhesion receptors composed of noncovalently associated α and β subunits. A number of integrin heterodimers are expressed by endothelial cells, including integrins α1β1, α2β1, α3β1, α5β1, α6β1, αvβ3, and αvβ5 [54]. While the short cytoplasmic tails of integrins lack intrinsic enzymatic activity, integrin engagement can stimulate intracellular signaling through the recruitment of kinases (including Src and focal adhesion kinase, FAK), adaptor proteins (Shc), and regulators of the actin cytoskeleton (Rho and Rac small GTPases; actin-binding proteins) to focal adhesion complexes, leading to activation of pathways regulating cell motility, morphology, proliferation, and survival [54,55]. Integrin signaling is essential for proper vascular development. Mice lacking endothelial expression of β1 integrin die by approximately E10.5, exhibiting defects in angiogenic remodeling and endothelial disorganization

[56,57,58]. β1 integrin function is also required for establishment of endothelial polarity and lumen formation [59].

In addition to regulating signaling through focal adhesion complexes, integrins also modulate the signaling of receptor tyrosine kinases in response to pro-angiogenic growth factor stimulation. Integrin αvβ3 has been shown to physically associate with activated VEGFR2, promoting VEGF-induced signaling and proliferation [60]. Moreover, function-blocking antibodies directed against either integrin αvβ3 or integrin αvβ5 inhibit angiogenesis induced by the angiogenic factors basic fibroblast growth factor (bFGF) and VEGF, respectively, through modulation of Erk activation [61,62,63].

1.3 Endothelial Cell Receptor Tyrosine Kinase Signaling Pathways

In addition to cell-cell and cell-matrix adhesions, endothelial function is regulated in part by a variety of soluble factors, including pro-angiogenic growth factors such as VEGF, bFGF, and the angiopoietins (Angpt) [64,65]. These factors signal through receptor tyrosine kinases (RTKs) to support endothelial cell proliferation, survival, and migration, as well as vascular stability. Among these factors, the VEGF/VEGFR and angiopoietin/Tie receptor systems are relatively endothelial cell-specific. In the following sections, I will describe the signaling pathways activated by VEGF and angiopoietins, as well as the role of these receptor systems in endothelial function both in normal physiology and pathology.

1.3.1 Vascular Endothelial Growth Factor (VEGF)/VEGFR Pathway

1.3.1.1 Ligands

Vascular endothelial growth factor (VEGF) has an important role in regulating angiogenesis during development, as well as during both physiological and pathological vascularization in adulthood [14]. The VEGF family of secreted glycoproteins includes five mammalian members, including VEGF-A, -B, -C, and –D and placenta growth factor (PlGF) [66]. Of the VEGF family members, VEGF-A (hereafter referred to as VEGF) is the primarily mediator of overall pro-angiogenic effects, while VEGF-C and VEGF-D regulate lymphangiogenesis [14,67]. VEGF exists in at least three distinct isoforms generated by alterative exon splicing, which are 189, 165, and 120 amino acids in length (in humans; the corresponding VEGF isoforms in mice are one residue shorter) [68]; these isoforms differ in their affinities for ECM binding [14]. Loss of a single *VEGF* allele in mice results in vascular defects and embryonic lethality [69,70], demonstrating the critical developmental role of this growth factor, as well as the requirement for precise control of VEGF expression levels for proper vascular development. Interestingly, intracrine signaling mediated by endothelial cell-derived VEGF is also required to support endothelial cell survival and maintain vascular homeostasis *in vivo* [71].

Hypoxia is an important regulator of VEGF expression [72]. Hypoxic conditions lead to stabilization of the transcription factor hypoxia-inducible factor-1α (HIF-1α), as well as the related HIF-2α protein, through inhibition of von Hippel-Lindau (VHL)-dependent HIF ubiquitination and proteasomal degradation. HIF-1α or HIF-2α

heterodimerize with HIF-1β (Arnt) to induce transcription of hypoxia-responsive genes including VEGF. Thus, hypoxia leads to increased VEGF-induced angiogenic sprouting, resulting in recruitment of new blood vessels to hypoperfused tissues. VEGF expression also is upregulated in response to a variety of growth factors, including epidermal growth factor (EGF), transforming growth factor β (TGFβ), fibroblast growth factor (FGF), and PDGF [14].

1.3.1.2 Receptors

VEGF can bind to either of two endothelial RTKs, VEGFR1 (also known as *Flt1*) and VEGFR2 (also known as *Flk1* in mice or *Kdr* in humans) [14]. The related VEGFR3 receptor binds to VEGF-C and VEGF-D. The VEGF receptors consist of an extracellular region containing seven immunoglobulin (Ig)-like domains, a single transmembrane domain, and an intracellular tyrosine kinase domain interrupted by a kinase-insert domain [73]. Binding of VEGF homodimers stimulates receptor homodimerization, resulting in *trans*-autophosphorylation of the receptor cytoplasmic domains and recruitment of signaling mediators. While VEGF binds to VEGFR1 with higher affinity than to VEGFR2, VEGF/VEGFR1 binding stimulates only modest receptor activation [74]. VEGFR2 thus is thought to mediate most of the pro-angiogenic effects of VEGF [75]. The 165 amino acid isoform of VEGF (VEGF165) also binds to the co-receptor neuropilin-1, which enhances VEGF/VEGFR2 binding and VEGF-induced proliferation and chemotaxis [76] and may regulate VEGFR2 trafficking and signaling [77].

Genetic depletion of *VEGFR2* in mice leads to embryonic lethality, with mutant embryos dying between E8.5 and E9.5 [10]. These embryos lack blood islands and organized blood vessels and exhibit diminished populations of hematopoietic progenitors, demonstrating a crucial role for VEGFR2 signaling in both vasculogenesis and hematopoiesis. Similarly, VEGFR1 knockout mice die during embryonic development but at later stages than VEGFR2 knockout mice (by E11.5), exhibiting abnormal vascular organization [78]. However, vascular development was normal in mice expressing a mutant form of VEGFR1 lacking the tyrosine kinase domain [79], suggesting that the role of VEGFR1 in vascular development is independent of its signaling activity.

1.3.1.3 VEGFR2 Intracellular Signaling and Endothelial Cell Responses

VEGF was first described both as a potent endothelial cell mitogen [80], as well as a permeability-inducing factor [81]. In fact, VEGF can stimulate a variety of pro-angiogenic responses in endothelial cells, including survival, proliferation, migration, permeability, and tube formation [75]. These varied endothelial cell responses result from the VEGF-induced activation of a diverse network of intracellular signaling mediators (**Figure 1.2**). VEGF binding results in VEGFR2 *trans*-autophosphorylation at tyrosines (Y) 1175 and Y1214 [82], as well as tyrosines 951, 996, 1054, and 1059 [66].

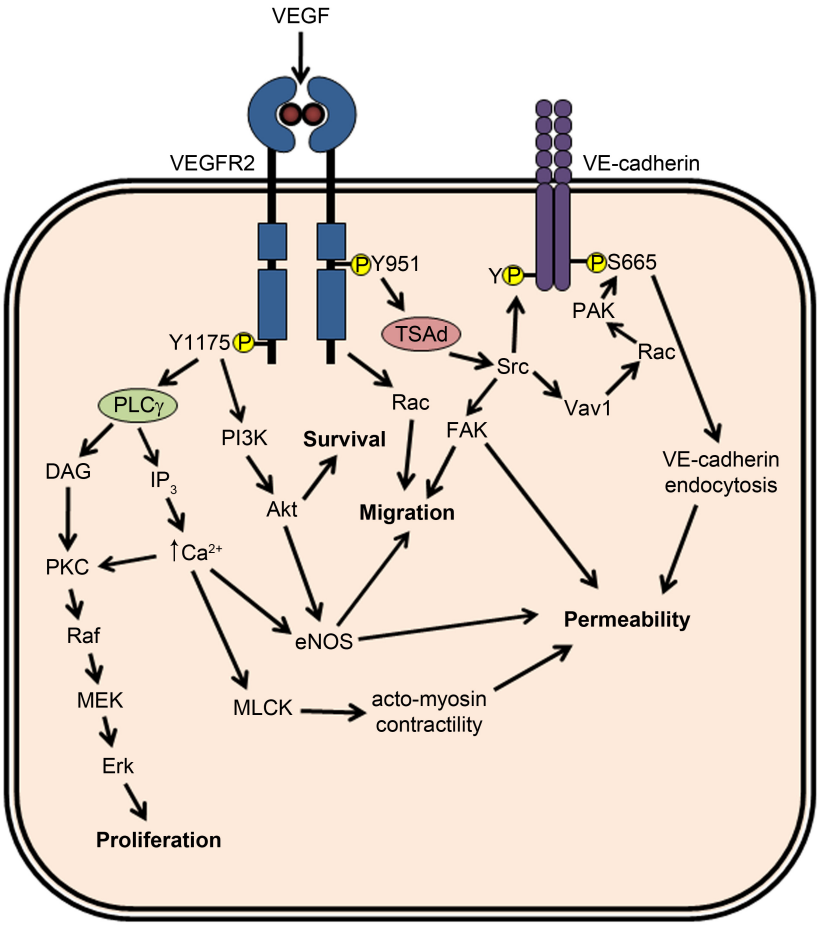

Figure 1.2 Major VEGF Signaling Pathways

Vascular endothelial growth factor (VEGF) homodimers bind to the receptor tyrosine kinase VEGF receptor 2 (VEGFR2), leading to receptor dimerization and *trans*-autophosphorylation, which results in recruitment and activation of a variety of intracellular signaling mediators. Activation of these signaling pathways results in endothelial cell responses including survival, proliferation, migration, and permeability. See text for description of VEGF-mediated signaling. Abbreviations: Y, tyrosine; PLCγ, phospholipase Cγ; PKC, protein kinase C; MEK, mitogen-activated protein kinase kinase; Erk, extracellular signal-regulated kinase; IP_3, inositol-1,4,5-trisphosphate; MLCK, myosin light chain kinase; PI3K, phosphoinositide 3-kinase; eNOS, endothelial nitric oxide synthase; TSAd, T-cell specific adaptor protein; FAK, focal adhesion kinase; PAK, p21-activated kinase; VE-cadherin, vascular endothelial cadherin.

VEGFR2 tyrosine phosphorylation results in recruitment of a variety of signaling proteins to the activated receptor, mediated by binding of their Src homology-2 (SH2) domains to autophosphorylated VEGFR2 tyrosine residues or binding to VEGFR2-associated adaptor proteins, including Grb2, Nck, and Shc [66,75]. As previously described, VEGF signaling also is modulated by interaction of VEGFR2 with VE-cadherin and integrin adhesion receptors [49,50,51,60].

The primary pathway by which VEGF supports endothelial cell survival is through activation of the PI3K [83,84]. PI3K catalyzes the production of phosphatidylinositol-3,4,5-trisphosphate (PIP_3), which mediates the membrane recruitment of the anti-apoptotic serine/threonine kinase Akt (protein kinase B), contributing to Akt activation. Akt inhibits apoptosis in part through phosphorylation and inactivation of pro-apoptotic regulators including BAD and caspase-9 [85,86]. VEGF stimulation also leads to the upregulation of anti-apoptotic proteins including Bcl-2 and A1, as well as the inhibitor of apoptosis proteins (IAPs) survivin and XIAP [87,88].

Phosphorylation of VEGFR2 (Y1175) is required for activation of phospholipase Cγ (PLCγ) [82], which contributes to activation of downstream pathways mediating endothelial cell proliferation and permeability [75]. PLCγ catalyzes the formation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate (PIP_2). DAG and increased cytosolic Ca^{2+} levels, resulting in part from IP_3-induced release of Ca^{2+} from intracellular stores, contribute to the activation of the

serine/threonine kinase protein kinase C (PKC), which mediates endothelial cell proliferation through activation of the Raf-MEK-Erk MAPK cascade [89].

The increased cytosolic Ca^{2+} levels also contribute to activation of endothelial nitric oxide synthase (eNOS) [75], which catalyzes the generation of nitric oxide (NO) from L-arginine. VEGF treatment further stimulates eNOS activation and NO production through Akt-mediated phosphorylation of eNOS on serine (S) 1179 (bovine residue; S1177 in human eNOS) [90]. eNOS activity contributes to VEGF-induced permeability and migration [91,92,93]. Endothelial cell-derived nitric oxide additionally serves an important function as a vasodilator, as well as an inhibitor of platelet activation and leukocyte adhesion [94]. Indeed, *in vivo* inhibition of VEGFR2 signaling is associated with increased blood pressure both in mice and in human cancer patients [95,96]. Increased Ca^{2+} levels also can contribute to activation of the Ca^{2+}/calmodulin-dependent enzyme myosin light chain kinase (MLCK) [97]. MLCK catalyzes phosphorylation of the myosin regulatory light chain (MLC2), which promotes acto-myosin contractility by increasing myosin ATPase activity [98]. Inhibition of MLCK activity inhibited VEGF-induced endothelial permeability *in vitro* [99].

Phosphorylation of VEGFR2 (Y951) leads to binding of the T-cell specific adaptor (TSAd) protein, which associates with the non-receptor tyrosine kinase Src [100]. This pathway has been linked to the regulation of VEGF-induced migration and permeability responses [100,101]. Importantly, knockout mice lacking expression of the Src family

kinases Src or Yes exhibited impaired VEGF-induced vascular permeability [102]. VEGF stimulation leads to Src-dependent tyrosine phosphorylation of VE-cadherin [103,104], which has been linked to destabilization of endothelial adherens junctions [105]. Src activation also has been shown to mediate endothelial permeability through phosphorylation of the guanine nucleotide exchange factor (GEF) Vav1, leading to the activation of the small GTPase Rac and its downstream target p21-activated kinase (PAK). PAK-mediated phosphorylation of VE-cadherin (S665) results in recruitment of β-arrestin, resulting in VE-cadherin internalization and dissolution of endothelial intercellular junctions [106]. In addition to its role in endothelial permeability, VEGF-induced Rac activation promotes endothelial cell migration [107]. In response to VEGF, Src also phosphorylates FAK on Y861, which promotes its recruitment to focal adhesions and was suggested to contribute to VEGF-induced endothelial migration [108]. FAK activation similarly is required for VEGF-induced permeability *in vivo* [109].

1.3.1.4 Role in Pathology

Although the adult vasculature is largely quiescent [29], the formation of new blood vessels can be stimulated as part of physiological processes including reproduction and wound healing [65]. While physiological angiogenesis is a tightly-regulated process, de-regulated angiogenesis leading to abnormal, excessive vascular growth is a key feature of several pathological conditions including tumor vascularization [64]. The acquisition of the ability to stimulate angiogenesis, the

"angiogenic switch," is an important step in the process of tumorigenesis [11], and in 1971, inhibition of angiogenesis was proposed as a strategy for cancer treatment [110].

Since that time, a multitude of studies have established a critical role for VEGF in mediating tumor vascularization [14]. VEGF is expressed in most human tumors, and it has been suggested that circulating VEGF levels may serve as a prognosis indicator for tumor progression [111]. Inhibiting the VEGF pathway has proven effective in inhibiting tumor vascularization and growth in numerous mouse models of cancer [14] and has shown clinical benefit in human cancer patients. Bevacizumab (Avastin), a humanized monoclonal antibody targeting VEGF-A, was the first anti-VEGF therapy to be approved by the United States Food and Drug Administration (FDA), after demonstration of improved survival in metastatic colon cancer patients treated with bevacizumab in combination with chemotherapy [112]. Additional anti-VEGF therapies, including the receptor tyrosine kinase inhibitors sunitinib, sorafenib, and pazopanib, have since been approved for the treatment of a variety of solid tumors [113]; these drugs inhibit VEGFR2 as well as other RTK targets. However, more recent reports have documented the development of tumor resistance to anti-angiogenic (including anti-VEGF) therapies, resulting from mechanisms including upregulation of alternative angiogenic factors, recruitment of pro-angiogenic bone marrow-derived cells, and most troublingly, increased tumor invasiveness [114].

Aberrant vascularization also is characteristic of several ocular diseases, including the neovascular (wet) form of age-related macular degeneration (AMD), as well as diabetic retinopathy [115], and can lead to severe vision loss or blindness. Elevated VEGF expression has been associated with these conditions [14,115], suggesting a role for VEGF activity in ocular neovascularization. Anti-VEGF therapies including bevacizumab and the related anti-VEGF humanized monoclonal antibody fragment ranibizumab (Lucentis) have improved clinical outcome in patients with neovascular AMD [116,117].

Elevated VEGF levels additionally can contribute to excessive, pathological vascular permeability [32]. Hypoxia-induced VEGF upregulation is observed following ischemic injury, including cerebral and myocardial ischemia [118,119]. While the induction of VEGF expression promotes eventual re-vascularization and recovery, VEGF also rapidly induces vascular permeability following ischemic events, which leads to edema and tissue damage [32]. Blocking VEGF pathway activity (or the activity of the downstream signaling mediator Src kinase) during this time period reduced tissue damage following stroke or myocardial infarction in mice [120,121,122].

Hyperpermeability is also a prominent characteristic of tumor microvessels [123], which may contribute to increased interstitial fluid pressure and impaired drug delivery, as well as tumor metastasis [124,125]. VEGF neutralization led to decreased vessel permeability and interstitial fluid pressure in mouse xenograft tumor models

[126,127]. In addition, tumor cells overexpressing VEGF displayed enhanced extravasation and lung metastasis in mice [128].

1.3.2 Angiopoietin/Tie Pathway

The angiopoietin-Tie receptor system regulates endothelial function during both physiological and pathological angiogenesis, as well as vascular homeostasis in the quiescent vasculature [129]. While VEGF signaling is necessary for initial vasculogenesis [10], angiopoietin signaling is important for subsequent vascular remodeling as well as for interaction of the endothelium with supporting mural cells [130,131].

1.3.2.1 Ligands

The angiopoietin (Angpt) family of secreted glycoproteins includes four members: Angpt1, Angpt2, Angpt3, and Angpt4 [129]. Of the angiopoietin ligands, Angpt1 and Angpt2 are the most well-characterized, while Angpt3 and Angpt4 are less-studied interspecies orthologs expressed in mice and humans, respectively [132,133]. Angpt1 and Angpt2 are highly similar structurally, each consisting of an N-terminal superclustering domain and coiled-coil motif, which mediate oligomerization and multimerization, as well as a C-terminal fibrinogen-related domain through which the angiopoietins bind to the Tie2 receptor [134,135].

Angpt1 is expressed primarily by pericytes and other mural cells [131,136] and can also be produced by monocytes [137]. In contrast, Angpt2 is produced by endothelial cells during development, but Angpt2 expression is not readily detectable in

quiescent vessels in adult tissues [134]. However, increased Angpt2 levels are observed at sites of inflammation or angiogenic remodeling [135], as mRNA expression of Angpt2 is upregulated in response to hypoxia or stimulation with cytokines or growth factors including VEGF [138,139]. In endothelial cells, Angpt2 protein is stored in unique endothelial cell-specific storage granules known as Weibel-Palade bodies [140], from which it can be rapidly mobilized in response to inflammatory mediators [141].

1.3.2.2 Receptors

Angpt1 and Angpt2 bind to the endothelial receptor tyrosine kinase Tie2 (tyrosine kinase with Ig-like and EGF homology domains-2), which is also known as TEK [134,136,142,143]. The Tie2 receptor consists of an extracellular portion including two N-terminal Ig-like domains followed by three EGF homology domains, a third Ig-like domain, and three fibronectin type III domains, as well as a single transmembrane domain and a C-terminal intracellular region consisting of a split tyrosine kinase domain [144]. Endothelial cells also express the related Tie1 receptor, which has a similar domain structure to Tie2, with 76% sequence identity in the intracellular domain, but only 33% identity in the extracellular region [143,145]. The Tie receptors are also expressed by some hematopoietic cells [129]. Unlike Tie2, Angpt1 and Angpt2 do not bind directly to Tie1 [134,136], which remains an orphan receptor. However, Tie1 may modulate angiopoietin signaling through formation of heteromultimers with Tie2 [146,147].

Binding of Angpt1 to Tie2 induces receptor clustering, resulting in *trans*-autophosphorylation of the receptor intracellular domain [136,148]. In contrast, treatment of endothelial cells with exogenous Angpt2 typically does not result in Tie2 phosphorylation/activation, and Angpt2 can block Angpt1-mediated Tie2 activation [134]. As a result of these findings, in combination with the similar phenotypes of *Tie2* knockout mice and mice overexpressing Angpt2 [134] (discussed in section 1.3.2.3), Angpt2 generally has been considered to act as a Tie2 antagonist [149,150]. However, several studies have shown that under certain conditions, endogenous Angpt2 or treatment with higher concentrations of exogenous Angpt2 can activate Tie2 [151,152,153], suggesting that Angpt2 rather functions as a context-dependent, partial Tie2 agonist. The differing potencies of Angpt1 and Angpt2 for mediating Tie2 activation may result from different receptor-binding affinities [153] or ligand multimerization [148]. A role for Tie1 in modulating Angpt2-mediated Tie2 activation also has been suggested, as lower doses of Angpt2 can stimulate Tie2 phosphorylation in cells lacking Tie1 expression [146].

In addition to the Tie2 receptor, both Angpt1 and Angpt2 can directly bind to integrins on endothelial and non-endothelial cells [154]. Interestingly, inhibition of Angpt2 was recently shown to impair vessel sprouting during postnatal retinal angiogenesis [155]. However, retinal endothelial tip cells express low levels of the Tie2 receptor [155], suggesting that Angpt2 promotes retinal angiogenesis independently of

Tie2. Angpt2 can directly bind αvβ3, αvβ5, and α5β1 integrins, albeit with lower affinity than to Tie2, and use of neutralizing antibodies targeting these integrins impairs Angpt2-mediated enhancement of vascular sprouting in both *in vitro* and *in vivo* assays [155]. Thus, Angpt2-induced processes can be mediated by Tie2-independent but integrin-dependent pathways.

1.3.2.3 Role in Vascular Development

Genetic depletion studies in mice have revealed crucial roles for the angiopoietins and Tie receptors in vascular development [130,131,156,157,158]. *Tie2*-null mice die by E9.5 to E10.5, exhibiting impaired branching and remodeling of the primary capillary plexus [130]. *Tie2*-deficient mice also display morphological defects in the cardiac endocardium and myocardium [131,159], as well as deficient recruitment of supporting mural cells [159]. Doxycycline-inducible *Tie2* inactivation also demonstrated a crucial pro-survival function of Tie2 in later stages of embryonic vascular development, as loss of Tie2 expression at approximately E11.5 rapidly leads to induction of endothelial cell apoptosis, resulting in loss of vascular integrity [160]. The orphan receptor Tie1 is required for normal vascular development, as *Tie1* knockout mice die between E13.5 and shortly after birth, exhibiting defective vascular integrity, edema, and hemorrhage [130,158]. Mice lacking Angpt1 expression also die embryonically (by E12.5), with mutant embryos exhibiting phenotypes similar to those observed in Tie2 knockout mice, including endocardial and myocardial defects, as well

as immature vascular structure and defective association with periendothelial cells [131]. These findings suggested that Angpt1 is the primary Tie2 agonist *in vivo*, and demonstrated that the Angpt1-Tie2 pathway serves an important function in regulating vessel remodeling and maturation during vascular development.

Transgenic mice overexpressing Angpt2 in the endothelium display phenotypes similar to, but more severe than, those seen in mice lacking Angpt1 or Tie2 expression [134]. Angpt2-overexpressing mice die between E9.5 and E10.5, exhibiting phenotypes including cardiac structural abnormalities, defective association between endothelial cells and underlying mesenchymal cells, and a disrupted, discontinuous vasculature [134]. The phenotypic similarities between these Angpt2 transgenic mice and *Angpt1* or *Tie2* knockout mice are consistent with Angpt2 functioning as a Tie2 antagonist in most contexts [149]. In contrast to the pronounced vascular defects and embryonic lethality observed upon loss of Angpt1 or Tie2, loss of Angpt2 expression in mice does not result in major abnormalities in embryonic vascular development [157]. However, *Angpt2* knockout mice (on a 129/J genetic background) display defects in patterning and function of the lymphatic vasculature, which result in severe chylous ascites and lead to the death of most mutant mice by two weeks of age [157]. Milder lymphatic defects are observed in *Angpt2*-null mice on the C57BL/6 genetic background, which survive to adulthood [161]. Interestingly, the lymphatic phenotype of *Angpt2*-null mice could be rescued by gene replacement with Angpt1, suggesting that Angpt2 likely functions as an

agonist in the lymphatic vasculature [157]. Mice lacking Angpt2 expression also display abnormal postnatal ocular vascular remodeling, including a lack of regression of the hyaloid vasculature and dramatically impaired retinal neovascularization. However, these defects are not rescued by Angpt1 expression [157].

In addition to its important role in vascular development, angiopoietin/Tie2 signaling may be required to maintain quiescence and homeostasis in the adult vasculature [129]. Tie2 phosphorylation/activation has been detected in the vasculature of all adult tissues examined [162]. However, the significance of this persistent Tie2 phosphorylation is unclear, as inducible global knockout of the Tie2 ligand Angpt1 did not affect late vascular development (after E13.5) or vascular homeostasis in the adult [163]. However, inducible *Angpt1* knockout mice display increased angiogenesis and fibrosis in a wound-healing model, as well as exacerbated diabetic kidney microvascular injury [163], suggesting an important role for Angpt1 in responses to injury or stress in the adult vasculature.

1.3.2.4 Tie2 Intracellular Signaling and Endothelial Cell Responses

Angiopoietin-Tie2 signaling influences a variety of endothelial cell processes, including survival, migration, and permeability, as well as inflammatory responses [129]. Following angiopoietin binding and receptor clustering, subsequent *trans*-autophosphorylation of the Tie2 intracellular domain results in recruitment and activation of a diverse set of signaling mediators (**Figure 1.3**).

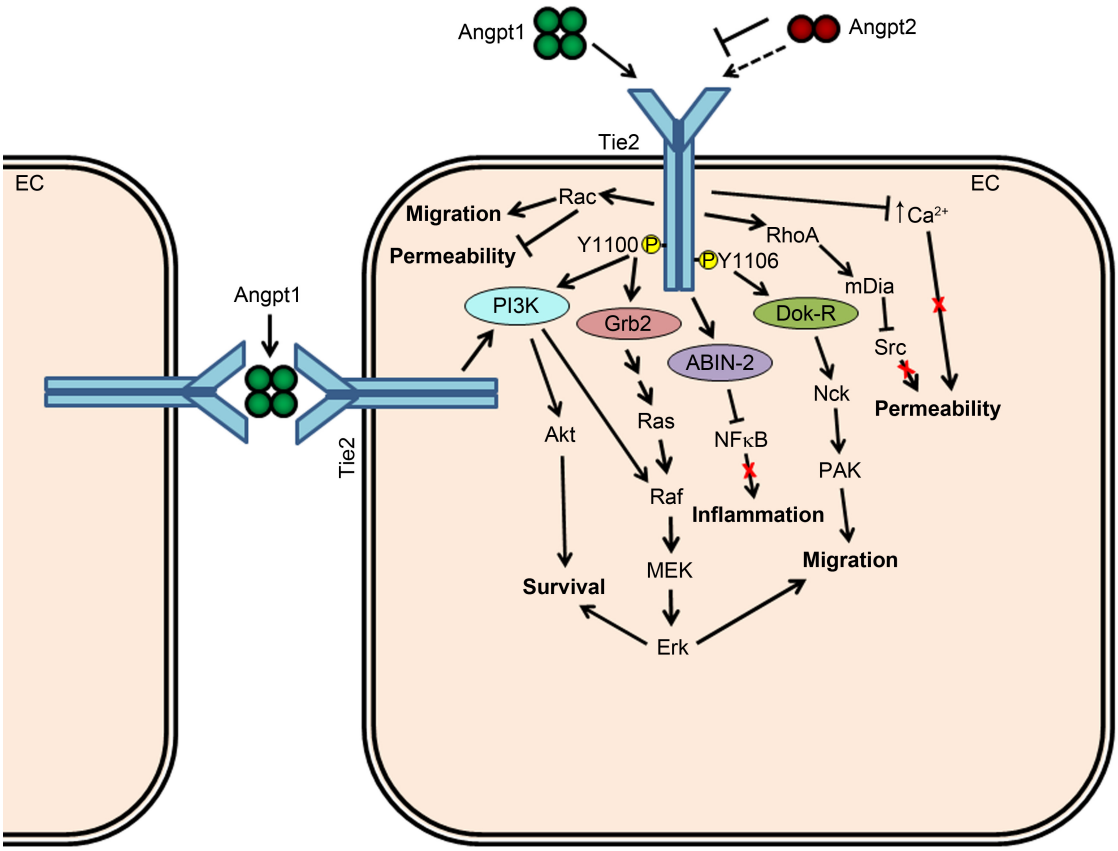

Figure 1.3 Major Angiopoietin Signaling Pathways

The secreted glycoprotein angiopoietin-1 (Angpt1), which is produced by perivascular cells including pericytes, binds to and activates the endothelial receptor tyrosine kinase Tie2. Angpt1 binding leads to activation of intracellular signaling pathways promoting endothelial cell survival and migration and inhibiting responses including permeability and inflammation. In contrast, angiopoietin-2 (Angpt2) predominantly is expressed by endothelial cells and functions as a context-dependent Tie2 antagonist or weak agonist. See text for description of angiopoietin (Angpt)-mediated signaling. Abbreviations: Y, tyrosine; PI3K, phosphoinositide 3-kinase; Grb2, growth factor receptor-bound protein 2; MEK, mitogen-activated protein kinase kinase; Erk, extracellular signal-regulated kinase; ABIN-2, A20-binding inhibitor of NFκB activation-2; NFκB, nuclear factor κB; Dok-R, downstream of tyrosine kinase-related protein; PAK, p21-activated kinase; mDia, diaphanous-related formin-1; EC, endothelial cell.

Several Tie2 autophosphorylation sites have been identified, including tyrosines 990, 1100, 1106, and 1111 (murine residues) [164,165,166]. The phosphorylated Tie2 intracellular domain interacts with a variety of SH2 domain-containing binding partners, including the adaptor proteins Grb2 (growth factor receptor-bound protein 2), Grb7, and Grb14, the tyrosine phosphatase Shp2 (SH2-containing protein tyrosine phosphatase), and the p85 subunit of PI3K [165], as well as the phosphotyrosine binding (PTB) domain-containing docking protein Dok-R (downstream of tyrosine kinase-related protein) [167].

Tie2 signaling has an important pro-survival function in endothelial cells. Loss of Tie2 expression has been linked previously to impaired endothelial cell survival, with increased apoptosis observed after inducible repression of Tie2 expression *in vivo* [160], as well as following *Tie2* knockdown *in vitro* [168,169]. Angpt1 treatment inhibits endothelial cell apoptosis induced by various cellular stresses [170,171,172]. These survival effects are mediated primarily by activation of the anti-apoptotic PI3K/Akt pathway [173,174], which results from direct recruitment of the p85 subunit of PI3K to phosphorylated Y1100 of Tie2 [175]. Angpt1 treatment leads to the upregulation of the IAP family member survivin, an effect which is dependent upon PI3K/Akt pathway activity [174]. *Survivin* upregulation may result from Akt-mediated inhibitory phosphorylation of the forkhead transcription factor FOXO1 (Forkhead box protein O1, also known as FKHR), which normally represses survivin expression in endothelial cells

[176]. A role for PI3K-dependent activation of the Raf-MEK-Erk MAPK pathway in Angpt1-mediated endothelial cell survival also has been demonstrated [177]. Activation of the Raf-MEK-Erk pathway additionally may be mediated through Grb2 and Shp2-dependent recruitment of the Ras GEF Sos to Tie2, leading to Ras-mediated activation of the serine/threonine kinase Raf [165,178]. In contrast to VEGF, however, Angpt1 is not a potent endothelial cell mitogen [136], suggesting that the Erk pathway may have different downstream effectors in VEGF- versus Angpt1-mediated signaling.

Interestingly, recent studies have demonstrated that Tie2-mediated activation of intracellular signaling pathways may be dependent upon cellular context and receptor localization [179,180]. Specifically, ECM-bound Angpt1 induced formation of Tie2 signaling complexes at cell-matrix contacts in sparse or migrating endothelial cells, while Angpt1 stimulated Tie2 translocation to cell-cell contacts and formation of Tie2-Tie2 *trans*-associated complexes in confluent endothelial cells. The activation of these distinct Tie2 complexes either at cell-cell or cell-matrix contacts was linked to preferential activation of the Akt or Erk pathways, respectively [179,180].

Angpt1/Tie2 signaling also stimulates endothelial cell migration [181], which requires PI3K activation [182]. However, an additional pathway regulating Angpt1-sitmulated migration has been identified, which is initiated by recruitment of the docking protein Dok-R to phosphorylated Y1106 on Tie2 [183]. Dok-R binds to the adaptor protein Nck, leading to the recruitment and activation of PAK, which in turn

promotes endothelial cell migration, likely through the regulation of cytoskeletal remodeling processes [184]. The Erk pathway also has been implicated in Angpt1-induced migration [179,185].

Along with its pro-angiogenic functions promoting endothelial cell survival and migration, Angpt1/Tie2 signaling contributes to several vascular protective and anti-inflammatory functions, including supporting endothelial barrier function [129]. Angpt1 treatment inhibits the induction of endothelial permeability by VEGF or thrombin *in vitro* [186,187], while transgenic overexpression of Angpt1 in mice stimulates formation of blood vessels resistant to VEGF-induced vascular leakage [188]. Several mechanisms underlie Angpt1-mediated endothelial barrier maintenance. Angpt1 treatment has been reported to lead to increased activation of the small GTPase Rac1, along with decreased RhoA GTPase activation [187,189]. Rac1 is known to support endothelial barrier function, through remodeling of cortical actin and stabilizing adherens junctions [190,191,192]. In contrast, RhoA activation promotes dissolution of endothelial cell-cell junctions by inducing acto-myosin contractility, which is mediated by increased phosphorylation of the myosin regulatory light chain following activation of the RhoA downstream target Rho kinase (ROCK) [30,193]. The negative regulation of RhoA by Angpt1 requires the activity of p190 RhoGAP (GTPase-activating protein) [189]. Interestingly, Angpt1 also has been reported to inhibit VEGF-induced permeability through the *activation* of RhoA [194]. In this pathway, Angpt1-induced RhoA activation

leads to sequestration of Src kinase, an important mediator of VEGF-induced permeability, through association of Src with the RhoA downstream target mDia (diaphanous-related formin-1). These conflicting reports raise the possibility that Angpt1 signaling may differentially regulate the activity of different RhoA GTPase pools or RhoA downstream effectors. Angpt1 additionally may inhibit endothelial permeability by inhibiting VEGF-induced influx of extracellular Ca^{2+} [195]. In contrast to the barrier-protective effects of Angpt1, Angpt2 is thought to destabilize the endothelial barrier. Angpt2 treatment leads to endothelial destabilization and detachment in three-dimensional co-cultures of endothelial cells and smooth muscle cells [196] and stimulates vascular leakage *in vivo* [197]. Angpt2 also enhances thrombin-induced endothelial permeability *in vitro* [187].

Angpt1 and Angpt2 similarly have opposing roles in the regulation of inflammatory responses. Angpt1 inhibits VEGF-induced expression of the adhesion proteins intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin, as well as impairs VEGF-induced leukocyte-endothelial cell adhesion [198]. Angpt1 also inhibits the induction of tissue factor expression by endothelial cells following stimulation with VEGF or the inflammatory cytokine tumor necrosis factor (TNF)-α [199]. The anti-inflammatory effects of Angpt1 are mediated by interaction of tyrosine-phosphorylated Tie2 with the zinc finger protein ABIN-2 (A20-binding inhibitor of NFκB activation-2), which inhibits nuclear factor κB (NFκB)-

mediated expression of inflammatory genes (including ICAM-1 and VCAM-1) [200]. In contrast, Angpt2 is required for inflammatory responses and sensitizes the endothelium to TNFα [161]. Notably, inducible endothelial overexpression of Angpt2 in mice results in myeloid cell recruitment to various organs, even in the absence of proinflammatory challenge, as well as increased responses to inflammatory stimuli [201].

1.3.2.5 Role in Pathology

Consistent with the opposing effects of Angpt1 and Angpt2 on endothelial barrier stabilization and inflammatory responses *in vitro* and in mice, a shift in angiopoietin balance, such that Angpt2 levels exceed Angpt1 levels, has been reported in a number of disorders involving vascular dysfunction, including diabetes, hyperoxic lung injury, and sepsis [202,203,204,205]. Elevated circulating Angpt2 levels have been documented in sepsis [206], and higher Angpt2 levels correlate with disease progression [135]. In addition, Angpt2 inhibition using function-blocking antibodies ameliorated disease progression in a mouse model of airway inflammation [207], and hyperoxia-induced acute lung injury was impaired in Angpt2-null mice [202], suggesting a potential active role for Angpt2 in progression and severity of disorders involving vascular dysfunction. Further, overexpression of Angpt1 has shown protective effects in mouse models of stroke, endotoxic shock, and fibrotic renal injury [208,209,210], indicating that restoration of Angpt1/Angpt2 balance may result in beneficial outcomes in these disorders. However, Angpt2 treatment also has shown positive effects in some

mouse disease models, including experimental models of sepsis and ischemia [211,212,213], which suggests that the role of Angpt2 in pathological conditions is highly context-dependent.

Both Angpt1 and Angpt2 have been implicated in tumor angiogenesis [214]. While not normally expressed in the quiescent adult vasculature [134], Angpt2 expression is upregulated in the vasculature of a variety of tumors [135]. Further, increased expression of Angpt2 correlates with poor prognosis of several cancers [215]. Based upon the expression patterns of VEGF and Angpt2 during various phases of tumor vascularization in a rat glioma model, it has been suggested that VEGF and angiopoietin signaling is highly coordinated during tumor angiogenesis, with the destabilizing ligand Angpt2 promoting vascular sprouting in the presence of VEGF, but leading to vessel regression in the absence of pro-survival signals [214]. Similar VEGF-dependent modulation of Angpt2 responses also has been observed in a rat ocular vascularization model [216]. Consistent with an important role for Angpt2 in tumor vascularization and disease progression, several studies have shown beneficial effects of Angpt2 inhibition (using function-blocking antibodies or peptibodies) in a variety of rodent tumor models, leading to decreased angiogenesis, tumor growth, and/or metastasis [217,218,219,220]. In addition, the Angpt1/Angpt2-neutralizing peptibody AMG386 demonstrated promising anti-tumor activity in combination with paclitaxel in a phase II clinical trial of patients with recurrent ovarian cancer [221], and AMG386 (in

combination with paclitaxel and carboplatin) is currently being investigated in a phase III trial (TRINOVA-1) for treatment of ovarian cancer. Interestingly, sustained systemic overexpression of Angpt2 led to disruption of vessel pericyte coverage, microvessel regression, and impaired tumor growth in a human colon carcinoma xenograft mouse model [222], suggesting that precise spatial and temporal regulation of Angpt2 expression levels is required for its tumor-promoting effects.

1.4 Abl Family of Non-Receptor Tyrosine Kinases

The Abelson (Abl) family of non-receptor kinases includes two mammalian members, Abl (Abl1, also known as c-Abl) and Arg (Abl-related gene, also known as Abl2), characterized by the presence of unique C-terminal actin-binding domains [223]. The Abl kinase initially was identified as the cellular homolog of the Abelson murine leukemia virus oncogene (*v-Abl*) [224,225] and later was implicated in the etiology of human chronic myelogenous leukemia (CML) as a result of the t(9;22) chromosomal translocation that produces the BCR-ABL1 fusion protein, which displays constitutive Abl kinase activity [226]. However, a growing body of literature also has demonstrated important functions for Abl and the closely-related Arg kinase in normal physiology, as these kinases play a role in intracellular signaling pathways activated by diverse extracellular stimuli, leading to regulation of cellular responses including cytoskeletal remodeling, proliferation, survival, adhesion, and migration [223,227,228].

1.4.1 Structural Properties

The Abl family kinases contain multiple structural and functional domains (**Figure 1.4**). The N-terminal portions of Abl and Arg are highly conserved, consisting of regulatory Src homology-3 (SH3) and SH2 domains, as well as the tyrosine kinase domain (SH1 domain) [229]; this domain structure is shared with other non-receptor tyrosine kinases, including Src family kinases [230,231]. Each of these domains display at least 90% amino acid sequence identity between Abl and Arg [229]. Alternative splicing, resulting in the usage of different 5′ exons, leads to the production of two major Abl isoforms [232]. Human Abl isoform 1b (isoform IV in mice) contains an N-terminal myristoylation site, which is not present in human Abl isoform 1a (murine isoform I) [232,233]. Similar myristoylated and non-myristoylated isoforms of Arg also have been identified [229]. The SH3 and SH2 domains, along with N-terminal myristoyl group attachment, have important roles in the intramolecular regulation of Abl kinase activation [234]. Abl also contains a region of approximately 80 amino acids located N-terminal to the SH3 domain; this "cap" region is thought to contribute to Abl intramolecular auto-inhibition [235].

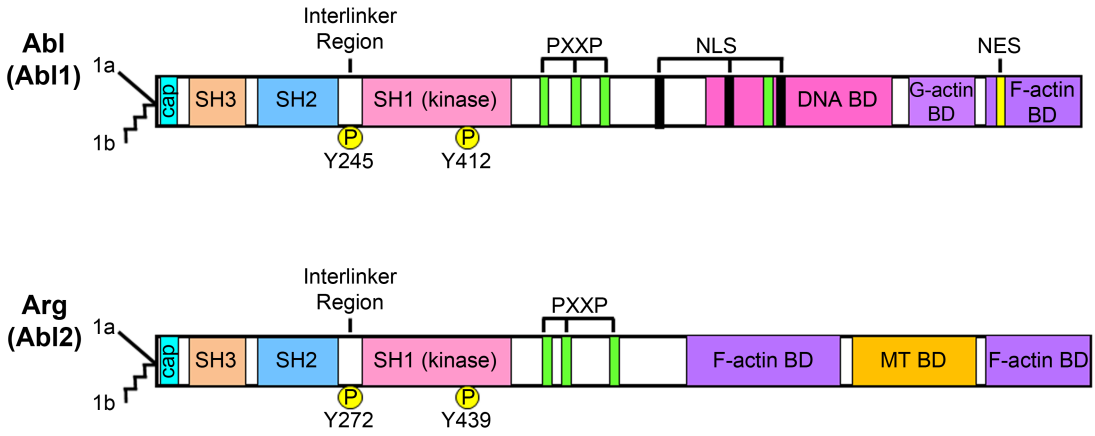

Figure 1.4 Structural Features of the Abl Family Kinases

Schematic of the major structural and functional domains of the Abl family of non-receptor tyrosine kinases (Abl and Arg). Alternative splicing leads to the generation of two major isoforms of each protein (1a and 1b); the 1b isoforms contain N-terminal myristoylation sites. The N-terminal regions of both Abl and Arg include highly-conserved Src homology-3 (SH3), SH2, and SH1 (tyrosine kinase) domains. Sites of regulatory tyrosine phosphorylation (Abl Y245/Arg Y272 in the SH2-SH1 interlinker region and Abl Y412/Arg Y439 in the kinase activation loop; numbering indicates position of tyrosine residues in Abl/Arg 1b isoforms) are indicated (yellow circles). Both Abl and Arg contain proline-rich sequences (PXXP motifs, green rectangles), as well as a C-terminal filamentous (F)-actin-binding domain (BD). Abl additionally contains a binding domain for monomeric globular (G)-actin, while Arg possesses a second, internal F-actin binding domain and a microtubule (MT)-binding domain. The Abl C-terminus also has three nuclear localization sequences (NLS, black rectangles), a nuclear export sequence (NES, yellow rectangle), and a DNA-binding domain, which are not found in Arg. Adapted from [236].

The SH3 and SH2 domains mediate interaction of the Abl family kinases with a variety of binding partners and substrates, through association with proline-rich regions and phosphorylated tyrosine residues on interacting proteins, respectively [223,228]. Protein-protein interactions are also mediated through proline-rich sequences (PXXP

motifs) found C-terminal to the tyrosine kinase domain in Abl and Arg, which are binding sites for interacting proteins containing SH3 domains [228]. The kinase domain of the Abl kinases preferentially phosphorylates tyrosine residues in the consensus sequence I/V/L-Y-X-X-P (I=isoleucine; V=valine; L=leucine; Y=tyrosine; X=any amino acid; P=proline) [237,238]. Numerous proteins have been identified as Abl/Arg binding partners and/or substrates, including signaling adaptor proteins and receptor tyrosine kinases, as well as regulators of cytoskeletal remodeling, DNA damage response, and apoptosis, among other processes [228].

In contrast to the highly-conserved N-terminal structural domains, Abl and Arg are more divergent in their C-terminal sequences, both from other non-receptor tyrosine kinases and from each other [228]. The C-terminal regions of Abl and Arg exhibit only 29% overall amino acid identity [229]. However, both Abl and Arg exhibit C-terminal binding domains enabling direct interaction with the actin cytoskeleton [239,240,241], which are not found in other non-receptor tyrosine kinases. Abl and Arg each contain a calponin homology (CH) domain at their extreme C-terminus that mediates binding to filamentous (F)-actin [239,240,241], while Abl also has a binding domain for monomeric globular (G)-actin [240]. Arg additionally possesses a second, internal F-actin binding domain (I/LWEQ domain), which is not found in Abl [241]. Both Abl and Arg have shown the ability to bundle F-actin *in vitro* [240,241]. Arg also contains a microtubule (MT) binding domain, which enables this kinase to mediate *in vitro* cross-linking of actin

filaments and microtubules [242]. Thus, the structural properties of the Abl family kinases enable their function in the regulation of processes involving cytoskeletal remodeling.

While Abl and Arg have a highly similar overall domain structure, the C-terminal region of Abl contains three nuclear localization sequences (NLS) not found in Arg [243,244], as well as a DNA-binding domain, which preferentially binds to A/T-rich DNA sequences [245,246]. Abl also possesses a functional nuclear export sequence (NES) [247], which enables nuclear-cytoplasmic shuttling of the Abl kinase in response to stimuli including integrin-mediated adhesion [247,248]. In contrast, Arg exhibits predominantly cytoplasmic localization [249]. Interestingly, a total of eight distinct Arg isoforms have been identified, resulting from alternative splicing at both the N- and C-termini [250]. One of these Arg isoforms localizes to the nucleus upon overexpression in COS-7 cells [251]; however, the mechanism underlying its nuclear localization and its functional consequences are unknown.

1.4.2 Regulation of Abl Family Kinase Activity

1.4.2.1 Intramolecular Regulation

The tyrosine kinase activity of the Abl kinases is tightly regulated in cells, in part through several auto-inhibitory mechanisms. The SH3 domain has a particularly important role in intramolecular inhibition of Abl kinase activity, as deletion of this region results in increased tyrosine phosphorylation of Abl, indicative of elevated Abl

kinase activity [252]. Structural studies have demonstrated that the SH3 and SH2 domains of Abl form a rigid clamp structure, which folds back onto the tyrosine kinase domain (on the side distal to the active site) to hold it in an inactive conformation [253]. This autoinhibitory association requires the binding of the SH3 domain to a polyproline sequence in the interlinker region connecting the SH2 and kinase domains [253], and mutation of interlinker proline residues leads to Abl kinase activation [252]. In this conformation, the SH2 domain closely docks with the distal surface of the C-terminal lobe (C-lobe) of the kinase domain, mediated by hydrogen bonding interactions [253]. In addition, the N-terminal myristoyl group present in some Abl/Arg splice variants binds to a hydrophobic pocket within the C-lobe. This binding acts to stabilize the inactive conformation of the kinase, by inducing a conformational change in the kinase domain to allow docking of the SH2 domain [253]. Mutation of the hydrophobic residues of the myristoyl binding pocket leads to increased Abl kinase activity [254]. Disruption of the SH3-SH2 clamp inhibitory function likely is mediated by binding of interacting proteins to either the SH3 or SH2 domain, resulting in displacement of the "clamp" from the kinase domain [227]; in this regard, treatment with phosphopeptides derived from known SH2 domain-binding Abl substrates activates Abl kinase activity *in vitro* [254].

1.4.2.2 Intermolecular Regulation

In addition to these auto-inhibitory interactions, the activity of the Abl kinases is regulated by interactions with a variety of cellular proteins, which act either to

positively or negatively regulate kinase activity [228]. Proteins which inhibit Abl kinase activity include the SH3 domain-binding proteins peroxiredoxin 1 (PRDX, also known as PAG) [255] and ABL-associated protein 1 (AAP1) [256], as well as proline-serine-threonine phosphatase-interacting protein 1 (PSTPIP1), which binds to the Abl kinase PXXP motifs and recruits the PEST-type protein tyrosine phosphatase (PTP-PEST, also known as PTPN12) to mediate dephosphorylation of Abl [257]. In the nucleus, the retinoblastoma protein tumor suppressor (Rb) binds to the ATP-binding lobe of the Abl kinase domain to inhibit kinase activity during the G1 phase of the cell cycle [258]; hyperphosphorylation of Rb relieves this inhibitory interaction, leading to Abl kinase activation upon entry into S phase. Binding to F-actin inhibits Abl kinase activity upon detachment of fibroblasts from the ECM, and deletion of the C-terminal actin-binding domain relieves this inhibition [259]. The activity of Abl and Arg also is inhibited by the phospholipid PIP_2, which binds to the Abl kinases, most likely in the kinase domain [260].

In contrast, association of Abl with distinct binding partners has been reported to increase Abl kinase activity. The Ras effector Rin1 (Ras and Rab interactor 1) interacts with both the SH3 and SH2 domains of Abl, leading to release of the Abl kinase from intramolecular inhibition [261]. The Crk adaptor protein also activates Abl kinase activity, through binding of the SH3 domains of Crk to the proline-rich motifs of Abl

[262]. In addition, Abl is activated through binding to the Nck adaptor protein [263] or to the docking protein Dok-R [264].

Pharmacological inhibition of the Abl kinases with small molecule inhibitors has proven useful for the therapeutic treatment of Abl-mediated pathologies as well as uncovering cellular processes dependent upon Abl family kinase activity. Imatinib (Novartis; also known as Gleevec or STI571) is a potent ATP-competitive inhibitor of the Abl kinases, as well as the BCR-ABL1 oncogene and the PDGF, colony-stimulating factor-1 (CSF-1), Kit, and discoidin domain (DDR) receptors [265,266,267,268]. Imatinib has proven extremely beneficial for the treatment of BCR-ABL1-driven CML [269]. However, development of imatinib resistance has led to the development of second-generation BCR-ABL1 (and Abl/Arg) kinase inhibitors, including nilotinib (Novartis; also known as Tasigna), which inhibits the Abl kinases with 10 to 20-fold increased potency compared to imatinib [270,271]. The sets of kinases targeted by imatinib and nilotinib are similar, as both of these inhibitors function by binding to and stabilizing the inactive conformation of the kinase domain [272]. More recently, novel allosteric Abl inhibitors have been identified, which inhibit Abl kinase activity through binding to the myristoyl binding site on the kinase domain [273,274]. These compounds, known as GNF-2 and GNF-5 (a GNF-2 analogue with improved pharmacokinetic properties [275]), display greater target specificity than imatinib or nilotinib and are not known to inhibit any kinases other than BCR-ABL1, Abl, and Arg [273,274].

1.4.2.3 Regulation by Phosphorylation

Along with release from intramolecular and intermolecular inhibition, full activation of the Abl and Arg kinases requires tyrosine phosphorylation, which stabilizes the active kinase conformation. Specifically, phosphorylation of Abl Y245 (in the interlinker region between the SH2 and kinase domains) and Y412 (in the activation loop of the kinase domain), or the analogous tyrosine residues in Arg, is required for maximal kinase activity (**Figure 1.4**) [276,277]. Mutation of Y412 to phenylalanine (F) decreased Abl activation by 90%, while the kinase activity of an Abl Y245F mutant was reduced by 50% [276]. Phosphorylation of these tyrosines can result from Abl/Arg autophosphorylation (occurring in *trans*) [276,277,278] or from Src-mediated phosphorylation [277,278,279]. Phosphorylation of Abl kinases on additional tyrosines, as well as serine or threonine phosphorylation, also can regulate kinase activity [228]. Abl kinase *trans*-autophosphorylation (and kinase activation) is enhanced by kinase oligomerization; in the BCR-ABL1 fusion protein, this is mediated by the N-terminal coiled-coil oligomerization domain of BCR [280].

Mechanisms exist which counterbalance the activating effects of phosphorylation-mediated Abl kinase activation. The Abl kinases are dephosphorylated by protein tyrosine phosphatases including PTPN12 (PTP-PEST) [257], leading to decreased kinase activity, and inhibition of tyrosine phosphatases increases Abl tyrosine phosphorylation [257,281] and kinase activity [278]. Abl kinase activity also is

downregulated through targeting of activated Abl kinases for ubiquitin-mediated proteasomal degradation [281].

1.4.2.4 Stimuli Leading to Abl Kinase Activation

The Abl kinases are activated in response to a wide range of both extracellular and intracellular stimuli and regulate an equally diverse set of cellular responses (**Figure 1.5**) [223,227,228,236]. The Abl kinases are activated downstream of numerous RTKs, including the receptors for PDGF [279], EGF [279], hepatocyte growth factor (HGF) [282], insulin-like growth factor 1 (IGF-1) [283], ephrin B4 [284], and agrin [285], leading to modulation of responses including receptor signaling, proliferation and survival, actin remodeling, motility, and invasion. PDGF-mediated Abl activation in fibroblasts requires both Src-mediated phosphorylation of Abl Y412 and PLCγ activation, which reduces levels of the Abl inhibitor PIP_2 through conversion to IP_3 and DAG [279,286].

The Abl kinases also are activated following engagement of immunoreceptors, including the B-cell receptor [287], T-cell receptor (TCR) [288], and Fcγ receptor (FcγR) [289]. Loss of Abl kinase activity impairs TCR-induced signaling, proliferation, and cytokine production [288,290], as well as TCR-stimulated integrin affinity maturation [291]. Abl kinases additionally are required for maximal FcγR-induced phagocytosis in macrophages [289]. Chemokine stimulation can lead to Abl kinase activation, as shown in response to stromal cell-derived factor 1α (SDF-1α) stimulation of T cells [292] or

breast cancer cells [293]. Chemokine-mediated Abl kinase activation is required for induction of chemotaxis and invasion in T cells and cancer cells, respectively.

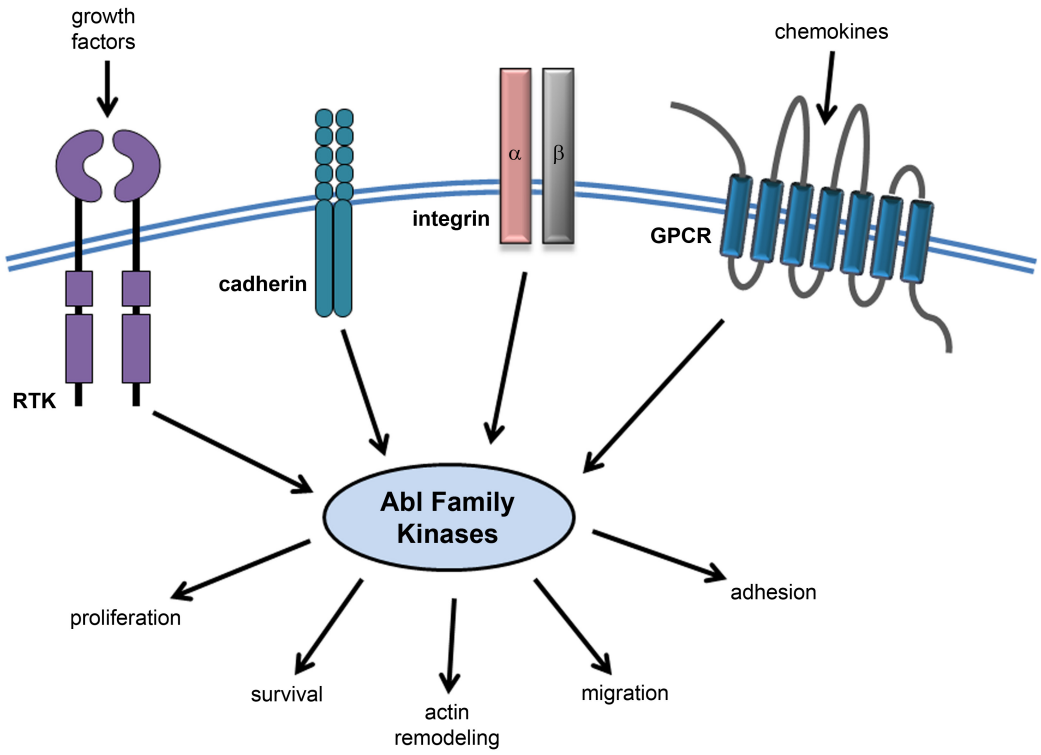

Figure 1.5 Abl Kinase Activating Signals and Downstream Responses

The Abl family kinases (Abl and Arg) are activated in response to a variety of extracellular stimuli, including growth factor and chemokine signaling, mediated by receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs), respectively. The Abl kinases also are activated following engagement of cadherin and integrin adhesion receptors. In response to these signals, Abl and Arg regulate numerous downstream cellular functions, including proliferation, survival, actin remodeling, migration, and adhesion. See text for description of Abl kinase activation mechanisms and effects of these kinases on cellular responses.

In addition, Abl kinase activity is modulated by adhesion receptors, including cadherins and integrins. In fibroblasts, integrin-mediated adhesion leads to Abl kinase

activation, along with shuttling of nuclear-localized Abl to the cytoplasm and recruitment of Abl to early focal contacts [248]. Abl kinases also are activated following cadherin engagement and are required for downstream Rac1 GTPase activation [294], which promotes actin polymerization and lamellipodial extension at nascent cell-cell adhesions [295].

Along with the extracellular stimuli described above, the Abl kinases are activated by intracellular stresses, including DNA damage and oxidative stress [223]. A number of DNA damage-inducing agents have been linked to activation of the nuclear Abl kinase pool, including ionizing radiation, cisplatin, and mitomycin C [296]. Abl activation in response to ionizing radiation is dependent upon activation of the ataxia telangiectasia-mutated (ATM) protein kinase, which phosphorylates Abl S465, leading to kinase activation [297]. Ionizing radiation-induced Abl kinase activation also may involve serine phosphorylation of Abl by the DNA-dependent protein kinase (DNA-PK) [298]. Oxidative stress induced by treatment with hydrogen peroxide results in activation of the cytoplasmic Abl kinase, which may involve activation of PKCδ [299,300].

1.4.3 Cellular Functions of the Abl Kinases

Consistent with the diverse set of stimuli which mediate Abl kinase activation, the Abl kinases have roles in the regulation of a variety of cellular processes, including cytoskeletal remodeling and modulation of cell-cell and cell-matrix adhesions

[223,227,228]. Here, I will briefly detail some of the major cellular roles of the Abl family kinases, along with the Abl kinase targets involved in regulating these processes.

1.4.3.1 Regulation of Actin-Dependent Processes

As previously described, Abl and Arg kinases possess unique actin-binding ability, which enables these kinases to modulate cytoskeletal remodeling in response to a variety of cellular stimuli [227]. The Abl kinases have been shown to localize to a variety of actin-rich cellular structures, including lamellipodial and filopodial cell protrusions [241,242,301,302], invadopodia [293,303], membrane ruffles [304], focal adhesions [248], stress fibers [240], and the leading edge of migrating cells [305], as well as at the immunological synapse [291] and in macrophage phagocytic cups [289]. Expression of the Abl kinases stimulates formation of membrane protrusions including lamellipodia and filopodia [241,242,306] and promotes neurite outgrowth and branching [307,308]. Conversely, loss of Abl kinase function impairs actin cytoskeletal remodeling processes including adhesion-induced lamellipodial protrusion [242] and PDGF-induced membrane ruffling [279] in fibroblasts, TCR- and chemokine-induced actin polymerization in T cells [292,309], and branching of primary cortical neurons [306,307].

The Abl kinases interact with and/or phosphorylate numerous cellular proteins involved in the regulation of actin cytoskeletal remodeling [228]. Among these targets are members of the Wiskott-Aldrich syndrome protein (WASP) and WASP-family verprolin-homologous protein (WAVE) family of actin-regulatory proteins. These

proteins associate with and promote the activity of the Arp2/3 (actin-related protein 2/3) actin-nucleating complex, which nucleates the formation of new branches from pre-existing actin filaments [310]. The Abl kinases phosphorylate several WASP/WAVE family members, including N-WASP, WAVE2, and WAVE3 [304,311,312]. Abl-mediated phosphorylation of WASP/WAVE proteins is required for actin remodeling processes including actin polymerization, lamellipodia formation, and *Shigella flexneri*-induced actin comet tail formation. Abl kinases also bind to and phosphorylate the Abl-interactor (Abi) adaptor proteins (Abi1 and Abi2) [313,314]. The Abi proteins, along with Nap1 (Hem2), Sra1 (PIR121), and HSPC300, associate with the WAVE proteins to form a complex (known as the WAVE complex) regulating Arp2/3-mediated actin nucleation [315]. In addition, the Abl kinases phosphorylate the actin-binding protein cortactin [316], which promotes Arp2/3-mediated actin polymerization and stabilization of actin filaments [227,310]. Abl kinase-mediated cortactin phosphorylation is required for actin-dependent formation of dorsal waves following PDGF treatment of fibroblasts [316] and EGF-induced invadopodia formation in cancer cells [303]. Loss of Abl kinase function also impairs cortactin and MT1-MMP localization to breast cancer cell invadopodia, leading to decreased ECM degradation and invasion [293].

The actin nucleation-promoting effects of the WASP and WAVE proteins are regulated by the small GTPases Cdc42 and Rac1, respectively [315]. Abl has been linked to Rac1 activation through phosphorylation of the GEF Sos-1 [317]. The Abl kinases also

regulate Rac1 activity through phosphorylation of the Crk/CrkL adaptor proteins; Abl-mediated Crk phosphorylation is required for Rac1 activation following adherens junction formation in mouse embryo fibroblasts (MEFs) [294]. Abl-mediated Crk phosphorylation also may negatively regulate Rac1 activity, by disrupting a complex containing Crk, $p130^{Cas}$, and the Rac1 GEF DOCK180 [318,319,320].

In addition to regulating Arp2/3-mediated actin polymerization, the Abl kinases regulate contractility of the actin cytoskeleton by modulating RhoA GTPase activity through phosphorylation of the RhoA negative regulator p190RhoGAP [321]. Abl/Arg-mediated phosphorylation stimulates p190RhoGAP inhibitory activity [321], leading to reduced levels of active RhoA and decreased acto-myosin contractility, as well as altered focal adhesion dynamics and fibroblast cell migration [322,323]. Increased RhoA activity, leading to stress fiber formation, acto-myosin contractility, and disrupted adherens junctions, also was observed following inhibition of the Abl kinases in epithelial cells [294]; however, no change in p190 RhoGAP phosphorylation were observed in this study, suggesting that the Abl kinases modulate RhoA activity and acto-myosin contractility through additional mechanisms.

1.4.3.2 Regulation of Cell-Cell and Cell-Matrix Adhesions

The Abl family kinases, which are activated following cadherin and integrin engagement [248,294], have important functions in the regulation of both cadherin-mediated cell-cell adhesion and integrin-mediated cell-matrix adhesion processes. Abl

kinases are required for adherens junction formation in both MEFs (N-cadherin) and epithelial cells (E-cadherin), as well as for maintenance of epithelial cell-cell junctions [294]. Conversely, expression of constitutively-active Arg was sufficient to promote formation of N-cadherin-mediated adherens junctions. This enhancement of cell-cell adhesion was dependent upon phosphorylation of the Abl kinase substrate Crk/CrkL, which was also required for activation of the Rac GTPase following cadherin engagement [294]. The Abl kinases also function downstream of active Rac as part of a positive feedback loop to promote adherens junction formation and stabilization [324]. In addition, Abl regulates β-catenin localization and dynamics (through phosphorylation of β-catenin Y667) and is required for proper remodeling of planar polarized junctions in *Drosophila* embryos [325].

The Abl kinases also have been implicated in integrin-mediated cell-matrix adhesion, with roles both in outside-in integrin signaling and inside-out modulation of integrin affinity. As previously discussed, the Abl kinase is activated and transiently recruited to nascent focal adhesions following integrin-mediated adhesion to a variety of extracellular matrix proteins [248]. The Abl kinases have demonstrated roles in a variety of integrin signaling responses, including promoting actin-based cellular protrusions [301], sustained cell-matrix adhesion, and cell spreading [326], as well as regulating focal adhesion dynamics [322,323]. Direct binding of the Arg kinase to the cytoplasmic tail of β1 integrin has been detected in excitatory neurons [327], and loss of Abl/Arg expression

in neurons leads to defects in integrin-mediated dendrite branch maintenance [307]. The Abl kinase also has been implicated in inside-out integrin signaling in T cells, as Abl expression is required for TCR-induced activation of the GTPase Rap1, which promotes integrin affinity maturation and increased extracellular matrix adhesion [291]. In contrast, expression of constitutively-active Arg kinase decreases Rap1 activation in epithelial cells, leading to impaired collagen-induced upregulation of β1 integrin protein [328].

1.4.4 *In Vivo* Roles of the Abl Kinases

Crucial roles for the Abl kinases have been demonstrated *in vivo*, through the use mouse mutants lacking expression of these kinases [329,330,331]. *Abl*-null mice, as well as mice expressing a mutant *Abl* allele which lacks the C-terminal one-third of the protein, have been generated [330,331]. In both cases, homozygous *Abl* mutant mice are runted and most die within one to two weeks after birth, depending on the genetic background. The primary phenotypes associated with global loss of Abl function (on a mixed genetic background) are thymic and splenic atrophy, reduced numbers of circulating B and T lymphocytes, and an increased susceptibility to infection, which were observed with variable penetrance [330,331]. Subsequent studies using conditional knockout mice have demonstrated a cell-autonomous role for the Abl kinase in T cell development and function [290,332].

Global *Arg* knockout mice have less severe defects than *Abl*-null mice; mice lacking *Arg* expression are viable, although somewhat runted at early ages, and display no histological abnormalities [329]. However, *Arg*-null mice demonstrate abnormal behavioral phenotypes including some motor skill and reflex defects, suggestive of improper neuronal function. Indeed, conditional *Abl* inactivation in neurons (in combination with global *Arg* inactivation) has revealed a requirement for the Abl kinases in the maintenance of dendrite branches in early adulthood, although initial dendrite arborization during development is normal in mutant mice [307] .

Interestingly, double-null mice lacking both *Abl* and *Arg* expression in all body tissues exhibit more severe phenotypes than mice lacking either kinase alone. Global *Abl*/*Arg*-null mice die embryonically by E11.0 and display defects in neural tube closure, as well as abnormal actin cytoskeletal architecture in neuroepithelial cells [329]. Large numbers of apoptotic cells also are observed in *Abl*/*Arg* double-null embryos, indicating a requirement for the Abl kinases in cell survival. Further, *Abl*-null; $Arg^{+/-}$ embryos, which die by E16.0, display peritoneal and pericardial hemorrhage and edema [329], suggestive of vascular defects. However, the specific vascular role of the Abl kinases had not yet been studied *in vivo* prior to the work described here.

1.4.5 Potential Vascular Roles for the Abl Kinases

As previously described, endothelial development and function require the coordination of a variety of cellular processes in response to signals including growth

factor-mediated RTK activation and engagement of adhesion receptors. The Abl kinases have been implicated in signaling responses to diverse stimuli in various cellular contexts, although limited studies have examined the role of these kinases in endothelial function. However, several previous investigations have suggested potential vascular functions for the Abl kinases; some of these studies will be reviewed in the following sections.

1.4.5.1 Angiogenesis

Elevated endothelial Abl kinase expression has been detected at sites of active angiogenic remodeling, both during development and in tumor blood vessels [333], suggesting the possible involvement of Abl in neovascularization. Further, imatinib has shown anti-angiogenic activity in several *in vivo* tumor models [334,335,336]. The anti-angiogenic activity of imatinib largely has been attributed to the inhibition of PDGF receptor activity, as PDGF signaling is necessary for endothelial cell interactions with supporting mural cells [335]. However, imatinib also can inhibit angiogenesis in a "porous chamber" subcutaneous implant mouse model, in which angiogenesis is driven by the pro-angiogenic factors VEGF and bFGF [266], suggesting the involvement of an alternative imatinib target in the angiogenic process. Indeed, during the course of the work described in this dissertation, a study was published describing a role for Abl in growth factor-driven angiogenesis [337]; this work has demonstrated a requirement for Abl in angiogenesis mediated by bFGF but not VEGF, attributed to involvement of Abl

in a signaling cascade downstream of the FGF receptor and integrin β3 [337]. The Abl kinase also has been implicated in VEGF-mediated signaling in human umbilical vein endothelial cells (HUVECs) [338], and *Abl*-knockout mice display reduced retinal neovascularization in response to hyperoxia [339].

1.4.5.2 Endothelial Barrier Function

Several studies have suggested a role for the Abl kinases in endothelial barrier function. Imatinib treatment has been shown to decrease interstitial fluid pressure in lung and colon cancer models, resulting in improved tumor oxygenation and drug delivery [340,341,342]. Imatinib treatment also reduces vascular permeability following administration of thrombolytic tissue plasminogen activator (tPA) in a murine model of ischemic stroke [343], suggesting a beneficial effect of imatinib on vascular barrier function. Pre-treatment with imatinib (or the more potent Abl kinase inhibitor nilotinib) similarly protects against pulmonary edema following lipopolysaccharide (LPS)-induced acute lung injury in mice [344], and imatinib treatment reduces vascular leakage and edema in a murine sepsis model [345]. While these protective effects have largely been attributed to imatinib-mediated inhibition of the PDGF receptor, recent studies have implicated the Abl kinases in both the promotion of endothelial barrier function by the bioactive lipid sphingosine-1-phosphate (S1P) [346] and the disruption of the endothelial barrier by VEGF and inflammatory mediators [345].

1.4.5.3 Cardiovascular Function

The Abl kinases have been linked to several additional aspects of cardiovascular function. *Abl*-null mice display decreased systolic blood pressure [347], and Abl has been implicated in signaling and constriction responses to phenylephrine and angiotensin II in arterial vascular smooth muscle [347,348]. The Abl kinase also has an important role in normal cardiomyocyte function and development, as *Abl*-null mice (on a C57BL/6J genetic background) exhibit perinatal lethality and heart enlargement, as a result of abnormally-increased cardiomyocyte proliferation [349]. However, cardiomyocyte-specific restoration of *Abl* expression largely rescues the observed cardiac hyperplasia, but does not restore viability, suggesting a critical role for the Abl kinase in additional cell types.

Notably, it has been reported that long-term treatment with imatinib causes severe congestive heart failure in a subset of CML patients, which has been attributed to the inhibition of the Abl kinase in cardiomyocytes, leading to endoplasmic reticulum (ER) stress and cell death [350]. Additional studies have reported left ventricular dysfunction and heart failure or increased levels of brain natriuretic peptide (BNP) in a subset of imatinib-treated cancer patients [351,352], as well as instances of interstitial lung disease of unknown origin [353]. Further, several case reports of cancer patients treated with the Abl kinase inhibitor nilotinib have detailed the occurrence of vascular occlusive events (infarction; peripheral arterial occlusive disease) in a subset of patients

[354,355,356], suggesting potential vascular dysfunction following Abl kinase inhibition. Similarly, adverse vascular events, including blood clots and narrowing of blood vessels, have been observed in over 10% of CML patients treated with the Abl kinase inhibitor ponatinib [357]. However, the role of the Abl kinases in the endothelium has not yet been examined *in vivo* using genetic models.

1.5 Objectives

The goal of the studies detailed in this dissertation was to evaluate the involvement of the Abl kinases in endothelial cell function *in vitro* and *in vivo*, using conditional genetic depletion of the Abl kinase in the endothelium, as well as specific Abl kinase pharmacological inhibition. Chapter 3 describes the generation and characterization of vascular phenotypes of endothelial *Abl* knockout mice. This work also examines the involvement of the Abl kinases in signaling cascades and endothelial cell responses downstream of a diverse set of cell surface receptors, including angiopoietin/Tie signaling and angiopoietin-1-mediated endothelial cell survival. Chapter 4 evaluates the involvement of the Abl kinases in endothelial permeability responses to mediators including VEGF, thrombin, and histamine. In all, these studies suggest both potential beneficial uses of Abl kinase pharmacological inhibitors, as well as deleterious vascular effects of targeting the Abl kinases; a discussion of these contrasting effects of Abl kinase inhibition and their possible implications for the clinical use of Abl kinase pharmacological inhibitors is included in Chapter 5.

2. Materials & Methods

2.1 Mice

Both *Abl*$^{flox/flox}$ mice and *Tie2-Cre* transgenic mice have been described previously (26, 34). *Abl*$^{flox/flox}$ mice were crossed into an *Arg*$^{-/-}$ background (13) and backcrossed six generations onto the C57BL/6 genetic background. *Abl*^{ECKO}; *Arg*$^{-/-}$ (mutant) embryos were obtained from timed matings of *Abl*$^{flox/flox}$; *Arg*$^{+/-}$; *Tie2-Cre*$^{+/-}$ males to *Abl*$^{flox/flox}$; *Arg*$^{+/-}$; *Tie2-Cre*$^{-/-}$ females. The presence of a vaginal plug was considered to be embryonic day (E)0.5. All animal procedures used in this study were reviewed and approved by the Duke University Institutional Animal Care and Use Committee (protocols #A183-07-07, A152-10-06, and A137-13-05) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council).

2.2 Cell Culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Lonza/Clonetics and used between passages 2 and 6. Human microvascular endothelial cells (HMVECs) immortalized with telomerase reverse transcriptase (hTERT) were provided by Xiao-Fan Wang (Duke University Medical Center) [358]. Endothelial cells routinely were cultured in microvascular endothelial growth medium-2 (EGM-2MV; Lonza). For experiments examining signaling responses to permeability-inducing factors, confluent HMVECs were serum-starved approximately 16 hours in endothelial

basal medium-2 (EBM-2) supplemented with 0.2% (wt/vol) bovine serum albumin (BSA). Cells were pre-treated with the Abl kinase inhibitors imatinib (10μM) or GNF-2 (15μM) or the Src kinase inhibitor su6656 (1μM) for one hour prior to treatment with VEGF (100ng/mL), thrombin (1U/mL), or histamine (100μM).

2.3 Inhibitors and Reagents

Imatinib (Gleevec/STI571) was a generous gift from Novartis and purchased from LGM Pharma. Nilotinib was purchased from Cayman Chemical. Recombinant human vascular endothelial growth factor (VEGF-A-165), recombinant human angiopoietin-1 (C-terminal 6-His tag), and anti-polyHistidine cross-linking antibody were purchased from R&D Systems. Recombinant bFGF was purchased from Invitrogen. GNF-2 and su6656 were purchased from Sigma. Thrombin from human plasma, histamine, thapsigargin, and PMA were purchased from Sigma.

2.4 Antibodies

Antibodies used for Western blotting included cleaved caspase-3, phospho-CrkL (Y207), phospho-Akt(S473), Akt, phospho-Erk(T202/Y204), phospho-PLCγ1 (Y783), phospho-VEGFR2 (Y1175), VEGFR2 (anti-human), phospho-MLC2 (S19), MLC2, and Rap1 from Cell Signaling; β-tubulin from Sigma-Aldrich; CrkL (C-20), Arg (9H5), Angpt2, VEGFR2 (anti-mouse, Flk-1), Erk, Lamin B, PLCγ1 (530), and Rap1GAP (H-93) from Santa Cruz; Abl (8E9) and Rac1 from BD Biosciences; Rho and Rac1 from Pierce (Thermo Scientific); phospho-Tie2(Y992) from R&D Systems; and mouse anti-α-catenin

from Zymed (Life Technologies). Mouse monoclonal antibody (Ab33) against Tie2 was a gift of Christopher Kontos (Duke University) (2). Antibodies used for both Western blotting and immunoprecipitation included VE-cadherin (C-19) from Santa Cruz and β-catenin from BD Biosciences. Antibodies used for immunohistochemistry included: CD31 (BD Biosciences), vWF and fibrin/fibrinogen (Dako), CD41 (eBioscience), alpha-smooth muscle actin (Sigma), and cleaved caspase-3 (Cell Signaling). VE-cadherin antibody (anti-Cadherin-5) from BD Biosciences was used for immunofluorescence.

2.5 Endothelial Cell Viability/Apoptosis Assays

Primary HUVECs ($5x10^3$ cells/well in 96-well plates) were cultured in serum-free medium (EBM-2 basal medium with 0.2% BSA) for 24 to 48 hours. Some cells were supplemented with either VEGF (100ng/mL) or bFGF (10ng/mL). Abl kinases were inhibited by treatment with imatinib (10μM). Cell viability in triplicate samples was assessed using CellTiter assay (Promega). In separate experiments, cells were lysed after 24 hours serum-starvation and cleaved caspase-3 levels examined by Western blotting. HUVECs expressing control miRNA or *Abl*/*Arg* miRNAs were serum-starved for 24 hours (96 hours post-miRNA infection). Caspase activity was assessed in triplicate samples using the Caspase-3/7-Glo assay (Promega) and normalized to total cell number. For Angpt1-mediated survival experiments, 200ng/mL Angpt1 (C-terminal 6-His tag) was pre-incubated with 20μg/mL anti-polyHistidine cross-linking antibody for 1 hour at 37°C in serum-free medium prior to treatment of HUVECs.

2.6 Mouse Endothelial Cell Isolation, Flow Cytometric Analysis, and Characterization of Abl Inactivation

E13.5 embryos (wild-type or Abl^{ECKO}; $Arg^{-/-}$) were disrupted by trituration and digested in 1mg/mL collagenase A solution (in low-glucose DMEM, supplemented with 1% penicillin-streptomycin, gentamicin, and 25μg/mL DNase I) for 45 minutes at 37°C. The cell suspension was then disrupted by passage (20X) through a syringe with attached 14Ga cannula and filtered through a 70μm cell strainer, followed by red blood cell lysis. Cells were incubated first for 15min on ice with anti-CD16/CD32 mouse Fc block (BD Biosciences), followed by staining for 30min with antibodies against CD105, CD45, and CD31 (all from eBioscience) or appropriate isotype control antibodies. After washing twice, cells were subjected to FACS, and $CD105^+/CD45^-/CD31^+$ endothelial cells collected. Retrovirus expressing Polyoma middle T antigen (PyMT) was used for endothelial cell immortalization (3). For flow cytometric analysis of endothelial cells, livers from E18.5 embryos (wild-type or Abl^{ECKO}; $Arg^{-/-}$) were similarly digested to a single-cell suspension, then labeled with antibodies against CD31, CD45, and Tie2 (CD202b, eBioscience) or appropriate isotype controls. Expression of each marker was evaluated by flow cytometry and Tie2 receptor levels examined in $CD31^+/CD45^-$ endothelial cells using FlowJo software (Tree Star Inc.). Endothelial cells were similarly isolated from hearts and livers from adult control and Abl^{ECKO}; $Arg^{+/-}$ mice, along with $CD105^-/CD45^-/CD31^-$ non-endothelial/non-hematopoietic cells. Cells were used directly for RNA isolation without culturing. $Oligo(dT)_{12-18}$-primed cDNAs were used for PCR

with the following primers: Abl Flox/Δ (forward) - 5′-GCC CTG GCC AGA GAT CCA TC-3′, (reverse) – 5′-TCC CTC AGG TAG TCC AGC AGG-3′; Abl exon 5 (forward) – 5′-GTA CGT GTC CTC CGA GAG CC-3′, (reverse) – 5′-CCT TCA GGA ACT CCT CCA CC-3′; Cre (forward) – 5′-CGA TGC AAC GAG TGA TGA GG-3′, (reverse) – 5′-CGC ATA ACC AGT GAA ACA GC-3′; β2M (forward) – 5′-ACC GGC CTG TAT GCT ATC CAG AAA-3′, (reverse) – 5′-GGT GAA TTC AGT GTG AGC CAG GAT-3′. The Abl Flox/Δ primers bind to sites within *abl* exons 4 and 6, respectively; the unrecombined abl^{flox} allele yields a 500 bp PCR product, while a recombined abl^{Δ} allele (lacking exon 5) yields a PCR product of approximately 200bp (4).

2.7 Flow Cytometry Analysis of Fetal Liver Cells

Livers were isolated from E15.5 wild-type and Abl^{ECKO}; $Arg^{-/-}$ embryos. Livers were maintained in Hank's Balanced Salt Solution without calcium or magnesium, supplemented with 10% (vol/vol) heat-inactivated FBS and 2mM EDTA, and were dissociated by gentle trituration. The resulting cell suspension was filtered through 70μm and 40μm cell strainers. Approximately 10^6 cells were used for surface staining of hematopoietic markers. Cells were first incubated with anti-CD16/CD32 mouse Fc block (BD Biosciences), followed by staining with antibodies against Kit and hematopoietic lineage markers (TER-119, GR1, B220, CD3, CD4, and CD8a) or erythroid markers (CD71 and TER-119) or appropriate isotype controls (all from eBioscience). After washing, cells

were analyzed by flow cytometry and expression of cell surface markers evaluated using FlowJo software (Tree Star Inc.).

2.8 Histological Analysis and Immunohistochemistry

Freshly-dissected mouse organs were imaged using a Zeiss Lumar.V12 stereoscope. For routine histology, mouse tissue samples were fixed in 4% (wt/vol) paraformaldehyde and embedded in paraffin; 5-μm sections were used for hematoxylin-eosin, Masson Trichrome, and Prussian blue staining, following standard protocols. Images were acquired using a Zeiss Axio Imager and Metamorph software. For FITC-wheat germ agglutinin (WGA) staining, sections were de-waxed and rehydrated, followed by proteinase K treatment (100μg/mL, 10 minutes at room temperature). Slides were washed with phosphate-buffered saline (PBS), then incubated with FITC-WGA (Sigma; 10μg/mL, 2 hours at room temperature). For immunohistochemical analysis, tissue samples were infiltrated with 30% (wt/vol) sucrose (in PBS), then fresh-frozen in tissue freezing medium (Tissue-Tek O.C.T.; Sakura Finetek); 10-μm sections were fixed/permeabilized in ice-cold acetone, blocked in 5% (vol/vol) normal goat serum, and stained overnight at 4°C with the indicated antibodies. Sections were then stained with Alexa Fluor-conjugated secondary antibodies (Invitrogen) and counterstained with Hoechst 33342 (0.5μg/mL). Images were acquired using a Zeiss Axiovert 200M fluorescence microscope and AxioVision software.

2.9 Viral Transduction

2.9.1 Retroviral Transduction

pLNCX vector and pLNCX/hTie2 retroviral constructs were kindly provided by Christopher Kontos, Duke University. Control (non-specific) and human *Rac1* shRNA oligos were cloned in pSuper-Retro-puro (pSR-puro) retroviral vector (OligoEngine). shRNA sequences (antisense) were as follows: control shRNA – AAA TGT ACT GCG CGT GGA G; *Rac1* shRNA – TTT TAC AGC ACC AAT CTC C. Retroviral constructs were transfected into 293T cells, along with pCL10A1 packaging vector, using FuGENE6 reagent (Promega). Retroviral supernatants were collected and filtered 48 hours post-transfection. HUVECs or HMVECs were incubated 16-24 hours with retroviral medium in the presence of 6μg/mL Polybrene. Transduction with pBabe-puro retroviral constructs (pBabe-puro vector; pBabe-puro/mAbl-WT; pBabe-puro/hRap1GAP) was performed similarly. HUVECs infected with hTie2-expressing retrovirus (or vector control) were cultured at least two days in 400μg/mL geneticin selection medium or sorted for Tie2 over-expression by FACS (mouse anti-hTie2 antibody; BioLegend) prior to microRNA (miRNA) lentiviral infection. HMVECs infected with control or *Rac1* shRNA retroviruses or with pBabe-puro vector-derived retroviruses were selected in 1μg/mL puromycin for at least two days.

2.9.2 Lentiviral Transduction

Lentiviral miRNA-mediated knockdown of *Abl*/*Arg* in HUVECs or HMVECs was conducted as previously described (5, 6). miRNA targeting sequences were as follows: control miRNA (GGTGTATGGGCTACTATAGAA); *Abl* miRNA (GGTGTATGAGCTGCTAGAGAA); *Arg* miRNA #1 (CCTTATCTCACCCACTCTGAA); *Arg* miRNA #2 (AGGTACTAAAGTGGCTCTGAG). *Abl* single knockdown in HMVECs was performed using a single lentiviral miRNA vector expressing 3 *Abl* miRNAs in tandem (miRNA Abl 6/2/1; generously provided by Owen Witte, University of California, Los Angeles, [359]). HUVECs were lysed for Western blotting or used for RNA isolation 96 hours post-miRNA infection. HMVECs were plated for signaling experiments and Transwell permeability assays 48 hours after miRNA viral transduction.

2.9.3 Cre Recombinase Adenoviral Transduction

In vitro Cre-mediated *Abl* deletion was performed using adenovirus expressing Cre recombinase (Ad5CMVCre-eGFP and Ad5CMV-eGFP control, obtained from the University of Iowa Gene Transfer Vector Core). Primary liver endothelial cells from $Abl^{flox/flox}$; $Arg^{+/+}$; $Tie2\text{-}Cre^{-/-}$ mice were incubated with adenovirus for 8 hours at a multiplicity of infection of 200. Lentiviral shRNA transduction was used for *Arg* knockdown in primary mouse endothelial cells. Lentiviral shRNA plasmid (pLKO.1 vector, The RNAi Consortium, (7)) targeting murine *Arg* was obtained from Open

Biosystems (Thermo Scientific), and control (non-specific) shRNA oligos were cloned into pLKO.1 vector. shRNA sequences (anti-sense) were as follows: control shRNA (TCGTAAGCCAGATAGTCATGC); *Arg* shRNA (TTGAGATAGGTCAATACCTGG). Transduced cells were selected in 1μg/mL puromycin for at least two days.

2.10 Lysis and Western Blotting

Cells were washed once with ice-cold phosphate-buffered saline (PBS), then lysed in radioimmunoprecipitation assay (RIPA) buffer [50mM Tris-HCl, pH 7.5; 150mM NaCl; 1% Triton X-100; 0.1% sodium dodecyl sulfate (SDS); 1% sodium deoxycholate; 0.05% NP-40; 5mM ethylenediaminetetraacetic acid (EDTA)] with protease/phosphatase inhibitors [0.1mM phenylmethane sulfonyl fluoride (PMSF); 1μg/mL aprotinin; 1μg/mL leupeptin; 10μg/mL pepstatin; 10mM β-glycerophosphate; 1mM sodium fluoride; 0.1mM sodium orthovanadate]. Alternatively, freshly-dissected mouse tissues were flash-frozen in liquid nitrogen, then homogenized in RIPA buffer containing protease/phosphatase inhibitors. Cell debris was removed by microcentrifugation, and protein concentrations were quantified using Bio-Rad *DC* protein assay reagents. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies in blocking solution containing either 5% (wt/vol) non-fat dry milk or 3% (wt/vol) BSA in Tris-buffered saline-Tween 20 (TBS-T), overnight at 4°C. Blots were washed three times with TBS-T, then incubated with horseradish peroxidase-coupled secondary antibodies

(Jackson ImmunoResearch or Santa Cruz) in blocking solution for one hour at room temperature. Blots were washed with TBS-T and developed using enhanced chemiluminescence (ECL) Western blotting detection reagent (Amersham/GE Healthcare). Western blots were quantified using ImageJ analysis software (NIH).

2.11 Real-time RT-PCR Array

RNA was isolated from control or *Abl*/*Arg*-knockdown HUVECs 120 hours post-miRNA infection, using a Qiagen RNeasy kit. 1μg each RNA was used for cDNA synthesis, using an RT^2 First Strand cDNA kit (SABiosciences). Gene expression was evaluated using the SABiosciences Human Endothelial Cell Biology Real-time RT-PCR Array, according to the manufacturer's protocol.

2.12 Real-time RT-PCR Analysis

RNA was isolated from HUVECs using an illustra RNAspin Mini RNA isolation kit (GE Healthcare) and cDNA synthesized using Oligo(dT)$_{12\text{-}18}$ primer and M-MLV reverse transcriptase (Invitrogen). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad). Primers used were as follows: *Tie2*/*Tek* (forward) – 5′-GGG CTA CAG ACT GGA GAA GC-3′, (reverse) – 5′-TGT CAA GGG TCT CCC ATG CC-3′; *Tie1* (forward) – 5′- ACC CTG GTG TGC ATC CGC AG-3′, (reverse) – 5′-GTG ATG TCC TCC CAC TCT AGC-3′; *Angpt1* (forward) – 5′-ACT GGG AAG GGA ACC GAG CC-3′, (reverse) – 5′-TGG AGG GGC CAC AAG CAT CAA-3′; *Angpt2* (forward) – 5′-GAC ACA CCA CGA ATG GCA TC-3′, (reverse) – 5′-CTG AAG GGT TAC CAA ATC CC-3′; *RPLP0*

(forward) – 5′-GGA CAT GTT GCT GGC CAA TAA-3′, (reverse) – 5′-GGG CCC GAG ACC AGT GTT-3′. Analysis was performed using a Bio-Rad CFX384 real-time machine and CFX Manager software. PCR reactions were performed in triplicate. Expression levels of each gene were normalized to the expression of the *RPLP0* ribosomal protein housekeeping control gene.

2.13 Biotinylation of Cell Surface Protein

Confluent HMVECs were serum-starved overnight (EBM-2 with 0.2% BSA), pre-treated for 1 hour with imatinib (10μM), then stimulated for 15 or 30 minutes with VEGF (100ng/mL) at 37°C. Cells were immediately moved to 4°C for the remainder of the procedure. Cell surface proteins were biotinylated with EZ-link Sulfo-NHS-SS-biotin (Thermo Scientific; 0.4mg/mL in PBS, with calcium and magnesium) for 30 min, followed by PBS washes and quenching of unreacted biotin in 50mM ammonium chloride in PBS with calcium and magnesium (2x5 minute washes). Following PBS washes, cells were lysed in RIPA Buffer (with protease/phosphatase inhibitors). For isolation of biotinylated cell surface proteins, equal amounts of each lysate (250μg) were incubated with high-capacity NeutrAvidin Agarose resin (Thermo Scientific) for 1 hour at 4°C with constant mixing. Beads were washed four times with PBS with 1% NP-40 and protease/phosphatase inhibitors, then boiled in 2X reducing SDS-PAGE sample buffer for analysis by western blotting.

2.14 Immunoprecipitation

Confluent HMVECs were serum-starved overnight (EBM-2 with 0.2% BSA), pre-treated for 1 hour with imatinib (10μM), then stimulated for 5 or 15 minutes with VEGF (100ng/mL). Cells were washed once with ice-cold PBS, then lysed in NP-40 lysis buffer (50mM Tris-HCl, pH 8.0, with 150mM sodium chloride and 1% NP-40) with protease/phosphatase inhibitors. Equal amounts of each lysate (300μg, pre-cleared) were incubated with either VE-cadherin (0.6μg) or β-catenin (0.5μg) antibodies overnight at 4°C with constant mixing, followed by incubation with Protein G Sepharose 4 Fast Flow beads (GE Healthcare Life Sciences) for 6 hours at 4°C. Beads were washed four times with NP40 buffer with protease/phosphatase inhibitors, then boiled in 2X reducing SDS-PAGE sample buffer for analysis by western blotting.

2.15 GTPase Activation Assays

Confluent HMVECs were serum-starved overnight (EBM-2 with 0.2% BSA), pre-treated with 10μM imatinib for 1 hour, then stimulated for 2 minutes with VEGF (100ng/mL) or thrombin (1U/mL). Activity of Rac1, Rap1, and Rho GTPases was examined using Active GTPase Pull-down and Detection kits (Pierce/Thermo Scientific), following the manufacturer's protocol.

2.16 Immunofluorescence

HMVECs were cultured to confluence on glass coverslips, then serum-starved approximately 16 hours in EBM-2 basal medium with 0.2% BSA. Cells were pre-treated

with either imatinib (10μM) or GNF-2 (15μM) one hour prior to treatment with VEGF (100ng/mL, 30 minutes) or thrombin (1U/mL, 5 minutes). Cells were washed with ice-cold PBS, then fixed 15 minutes in 4% paraformaldehyde (in PBS) and permeabilized 15 minutes in 3% BSA in PBS with 0.05% sodium azide and 0.05% Triton X-100. VE-cadherin primary antibody (anti-Cadherin-5; BD Biosciences) was added to coverslips at 1:200 dilution in blocking buffer (3% BSA in PBS with 0.05% sodium azide) for one hour at room temperature. Following PBS washes, Alexa 568- or Alexa 488-coupled goat anti-mouse IgG was added at 1:250 dilution in blocking buffer for one hour at room temperature, followed by DNA counterstaining with Hoechst 33342 (0.5μg/mL, 5 minutes). Images were acquired using a Zeiss Axiovert 200M fluorescence microscope and AxioVision software (Rel. 4.8).

2.17 Transwell Permeability Assay

Transwell supports (6.5mm diameter, 0.4μm pore size, polyester membranes; Corning) in 24-well tissue-culture plates were pre-coated with 0.02mg/mL bovine fibronectin (Sigma) for one hour at 37°C, prior to seeding of HMVECs at $2.7x10^4$ cells per Transwell. HMVECs were cultured in Transwells for three days at 37°C to allow formation of a confluent monolayer. Cells were then pre-treated with Abl kinase inhibitors in serum-free medium (EBM-2 with 0.2% BSA) for one hour prior to permeability assay. Fluorescein-labeled dextran (anionic, molecular weight 40kDa; Invitrogen/Life Technologies) was added to the top chamber of each Transwell at

1mg/mL, followed by treatment with permeability-inducing factors. Each treatment was performed in triplicate wells. At the indicated times, 50μL samples were removed from the bottom chamber of each Transwell and replaced with 50μL fresh culture medium. The collected samples were diluted 20-fold with PBS and fluorescence measured (excitation 485nm/emission 535nm) using a VICTOR^3 plate reader (PerkinElmer) and Wallac 1420 Workstation software.

2.18 In Vivo Permeability Assays (Modified Miles Assay)

2.18.1 Abl Kinase Inhibition

In vivo vascular permeability was examined using a modified Miles assay [360], examining leakage of Evans blue dye as an indicator of albumin extravasation. Approximately 12-week-old female athymic nude mice were anesthetized with 100mg/kg ketamine and 10mg/kg xylazine. Evans blue dye (Sigma; 30mg/kg in 100μL sterile saline) was administered by tail vein injection, followed by intradermal injections of PBS and VEGF (100ng in 30μL PBS) into the back skin, co-injected with either vehicle or either of two Abl kinase inhibitors (imatinib or GNF-2, 15 μM). Fifteen minutes after intradermal injections, mice were humanely euthanized by CO_2 inhalation and the back skin surrounding each injection site dissected, blotted dry, and weighed. Evans blue dye was extracted from skin tissue samples in formamide (Sigma) overnight at 56°C, and absorbance measured at 620nm. Amounts of extracted Evans blue dye were calculated

based on a standard curve of Evans blue dye in formamide and normalized to skin tissue sample weight.

2.18.2 Endothelial *Abl* Knockout Mice

The modified Miles assay described above was also performed using approximately 16-week-old endothelial *Abl* knockout mice ($Abl^{flox/flox}$; $Arg^{+/-}$; $Tie2\text{-}Cre^{+/-}$, referred to as Abl^{ECKO}; $Arg^{+/-}$) and age- and sex-matched $Arg^{+/-}$ controls ($Abl^{flox/flox}$; $Arg^{+/-}$; $Tie2\text{-}Cre^{-/-}$). Mice were anesthetized with 100mg/kg ketamine and 10mg/kg xylazine, followed by intravenous administration of 30mg/kg Evans blue dye. Back hair was shaved using mouse clippers prior to intradermal injections of PBS and VEGF (100ng in 30μL PBS). Mice were euthanized 15 minutes after VEGF administration, and Evans blue dye was extracted and quantified as described above.

2.19 Analysis of Intracellular Ca^{2+} Levels

HMVECs were cultured to confluence on 35mm glass bottom microwell dishes (MatTek Corporation; Ashland, MA, USA), then serum-starved overnight in EBM-2 basal medium with 0.2% BSA. Cells were loaded with 3μM cell-permeant Calcium Green-1, AM fluorescent Ca^{2+} indicator (Molecular Probes/Life Technologies) in the presence of 0.1% Pluronic F-127 in serum-free medium for 45 minutes at room temperature. Cells were washed three times with Hank's Balanced Salt Solution (HBSS; with calcium and magnesium, without phenol red), then pre-treated with imatinib (10μM) or GNF-2 (15μM) in HBSS for 1 hour. Intracellular Ca^{2+} levels, as assessed by

Calcium Green-1 fluorescence, were measured by live cell imaging using a Zeiss Axio Observer Z1 fluorescence microscope (GFP filtercube: BP 470/40, FT 495, BP 525/50) and MetaMorph software. Images were acquired using four microscope fields per treatment. Three images were acquired prior to addition of permeability-inducing factors, for assessment of baseline intracellular Ca^{2+} levels. Images were acquired for 10 minutes (at 20 second intervals) following addition of VEGF (100ng/mL), thrombin (1U/mL), or histamine (100μM) in HBSS. Average fluorescence intensity (with baseline subtracted) was measured on a single-cell basis for 35 cells per treatment, using Metamorph software.

2.20 Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 or 6 software. Comparisons of two groups were performed using Student's *t* tests (two-tailed). Comparisons involving multiple groups were evaluated using one-way ANOVA, followed by Bonferroni post-tests. Two-way ANOVA, followed by Bonferroni post-tests, was used to evaluate differences in HUVEC survival between drug treatments over time, changes in mRNA expression in control vs. *Abl*/*Arg* knockdown cells, and changes in HMVEC permeability in multiple treatment groups over time. Survival of embryos of various genotypes was evaluated by log-rank (Mantel-Cox) test. For all tests, $P<0.05$ was considered statistically significant.

3. Abl Kinases Are Required for Vascular Function, Tie2 Expression, and Angiopoietin-1-Mediated Survival

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3.1 Introduction

Disruption of vascular homeostasis plays a key role in pathological conditions including atherosclerosis, cancer, diabetes mellitus, and inflammatory arthritis [2,3]. Endothelial function is regulated, in part, by a variety of vascular growth factors, including VEGF and the angiopoietins (Angpt) [64]. These factors signal through receptor tyrosine kinases to support endothelial cell proliferation, survival, migration, and vascular stability. The angiopoietins, of which Angpt1 and Angpt2 are most well-characterized, signal through the Tie2 receptor. Angpt1 is the primary Tie2 stimulatory ligand, while Angpt2 functions as a context-dependent Tie2 antagonist or agonist [361,362]. While VEGF signaling is necessary for initial vasculogenesis, angiopoietin signaling is important for subsequent vascular remodeling as well as for interaction of the endothelium with supporting mural cells [70,130]. Vascular growth factor signaling also functions to maintain homeostasis in the quiescent vasculature [71,129]. Thus, delineating the intracellular signaling mechanisms that mediate endothelial responses to these factors has important implications for understanding vascular homeostasis as well as cardiovascular diseases.

The Abl family of non-receptor tyrosine kinases, which includes the Abl (Abl1) and Arg (Abl2) kinases, has roles in diverse cellular processes including proliferation, survival, adhesion, and migration [223]. These kinases are transiently activated and mediate cytoskeletal remodeling downstream of several growth factor receptors and following cadherin and integrin engagement [223,294]. Abl is activated constitutively as a result of the t(9;22) chromosomal translocation that produces the BCR-ABL1 fusion protein, the causal agent in CML [226]. Global *Abl*/*Arg*-null mice die by E10.5, exhibiting hemorrhage and pericardial edema, suggesting a role for these kinases in vascular development [329]. It was reported that long-term treatment with imatinib (Gleevec/STI571), a pharmacological inhibitor of the Abl kinases (as well as Kit and the platelet-derived growth factor receptor) caused severe congestive heart failure in a subset of CML patients [350]. This cardiotoxicity was attributed to Abl inhibition in cardiomyocytes, leading to endoplasmic reticulum stress and cell death. Similarly, global *Abl* deletion (C57BL/6J genetic background) led to cardiomyocyte dysfunction, heart enlargement, and perinatal lethality [349]. However, cardiomyocyte-specific restoration of *Abl* expression did not rescue viability [349], suggesting a critical role for the Abl kinase in additional cell types. Notably, several case reports of patients treated with the second-generation BCR-ABL1 kinase inhibitor nilotinib have detailed the occurrence of vascular occlusive events [354,355], suggesting potential vascular dysfunction following Abl kinase inhibition. *In vitro* studies have demonstrated a

requirement for the Abl kinases in mediating both endothelial barrier-promoting effects of sphingosine-1-phosphate and barrier-disrupting effects of VEGF and inflammatory mediators [345,346]. However, the role of the Abl kinases in the endothelium has not yet been examined using genetic models.

Here, we demonstrate a crucial role for the Abl kinases in the vasculature using endothelial *Abl* knockout mice. Loss of endothelial Abl kinases resulted in lethality at late embryonic and perinatal stages of development, with focal regions of vascular loss and tissue necrosis/apoptosis. Further, we demonstrate increased endothelial cell apoptosis in these embryos, as well as in *Abl*/*Arg*-knockdown endothelial cells *in vitro*. Notably, our studies reveal an unexpected link to angiopoietin/Tie2 signaling, with loss of endothelial Abl kinases leading to decreased Tie2 expression, diminished Tie2 receptor signaling, and loss of Angpt1-mediated survival. Further, we find that Abl kinases are activated by Angpt1/Tie2 signaling. Together, these findings reveal bi-directional signaling linking Abl kinases and Tie2, which is critical for endothelial cell survival and function.

3.2 Results

3.2.1 Embryonic Lethality of Abl^{ECKO}; $Arg^{-/-}$ Mice

To evaluate the vascular function of the Abl kinases, we generated mice with endothelial inactivation of the Abl kinase by crossing mice carrying a floxed *Abl* allele ($Abl^{flox/flox}$ mice) on an $Arg^{-/-}$ background to *Tie2-Cre* mice. In these mice, Cre-mediated

recombination results in excision of a 2.2kb genomic fragment containing *abl* exon 5, yielding minimal levels of a truncated, kinase-inactive Abl protein [290,307]. A near-complete loss of both *Abl* mRNA and protein was observed in endothelial cells of both embryos and adult mice (**Figure 3.1A-D**). No Cre-mediated recombination or Abl depletion was observed in non-endothelial/non-hematopoietic cells (**Figure 3.1B-D**).

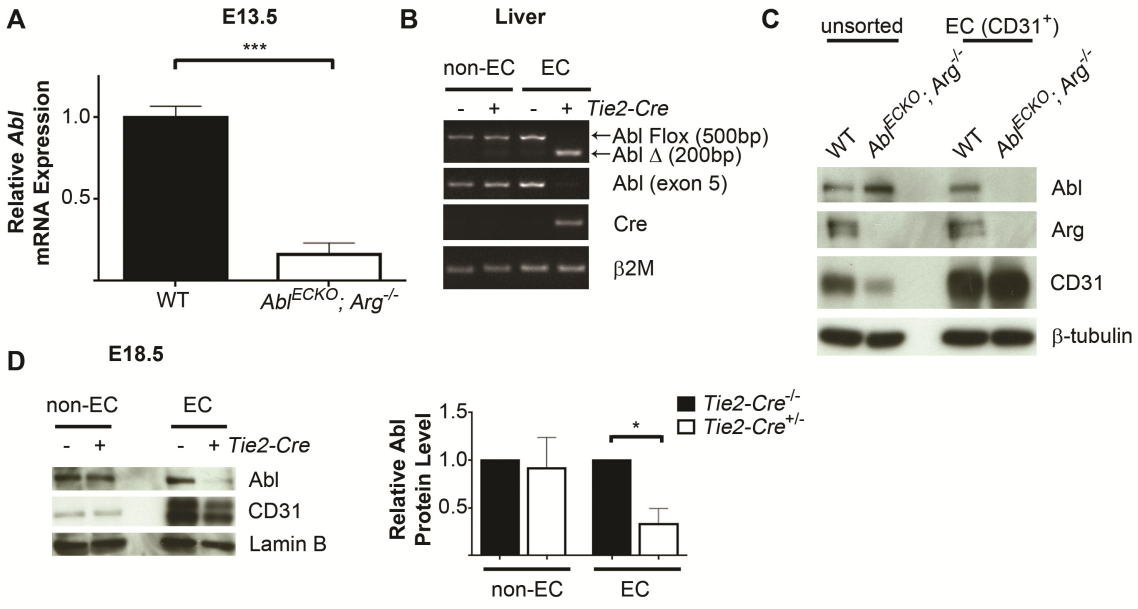

Figure 3.1 Analysis of Abl mRNA and Protein Levels in *Abl*^{ECKO} Mouse Endothelial Cells

(**A**) Assessment of *Abl* knockdown levels in *Abl*^{ECKO}*; Arg*$^{-/-}$ embryos. Endothelial cells (CD105^{+}/CD45^{-}/CD31^{+}) were isolated from E13.5 *Abl*$^{flox/flox}$*; Arg*$^{+/+}$*; Tie2-Cre*$^{-/-}$ (wild-type, WT) and *Abl*^{ECKO}*; Arg*$^{-/-}$ (mutant) embryos. *Abl* mRNA expression was assessed by real-time RT-PCR. Data are shown as means +/- SD, n=5-6 embryos/genotype. (**B**) Endothelial-specific *Abl* inactivation in livers from *Abl*^{ECKO}*; Arg*$^{+/-}$ adult mice. RNA was isolated from endothelial (EC; CD105^{+}/CD45^{-}/CD31^{+}) and non-hematopoietic/non-endothelial (non-EC; CD105^{-}/CD45^{-}/CD31^{-}) liver cell populations from *Abl*^{ECKO}*; Arg*$^{+/-}$ (+ *Tie2-Cre*) and *Arg*$^{+/-}$ control (- *Tie2-Cre*) mice, and Cre-mediated recombination of *abl* exon 5 was assessed by RT-PCR. Deletion of *abl* exon 5 was only observed in endothelial cells (200bp band in top panel vs. 500bp band in non-recombined allele). (**C**) Analysis of Abl and Arg protein depletion in PyMT-immortalized cells from WT and *Abl*^{ECKO}*; Arg*$^{-/-}$

embryos, either prior to (unsorted) or after sorting for $CD31^+$ endothelial cells (EC). (**D**) Abl protein depletion specifically in endothelial (EC; $CD105^+/CD45^-/CD31^+$) cells, rather than non-hematopoietic/non-endothelial (non-EC; $CD105^-/CD45^-/CD31^-$) cells, from E18.5 $Abl^{flox/flox}$; $Tie2\text{-}Cre^{-/-}$ and $Abl^{flox/flox}$; $Tie2\text{-}Cre^{+/-}$ embryos, quantified in right panel (means +/- SD, n=3). (*P<0.05; ***P<0.001).

Strikingly, loss of the endothelial Abl kinases resulted in significant lethality. The majority of endothelial *Abl*/*Arg*-null pups ($Abl^{flox/flox}$; $Arg^{-/-}$; $Tie2\text{-}Cre^{+/-}$, hereafter referred to as Abl^{ECKO}; $Arg^{-/-}$ or mutant) died at birth, with 90% mortality by the end of postnatal day 1 (**Figure 3.2A**). Further, the total number of Abl^{ECKO}; $Arg^{-/-}$ mice born was reduced (6.5%) compared to the expected Mendelian ratio (12.5%), suggesting that approximately 50% died during embryonic development. While mutant embryo viability largely was unaffected at earlier stages of cardiovascular development, a decreased number of Abl^{ECKO}; $Arg^{-/-}$ embryos was observed at later stages (E17.5-E18.5), and mutant embryo viability was decreased (**Figure 3.2B**). In contrast, no decrease in viability of $Arg^{-/-}$ embryos ($Abl^{flox/flox}$; $Arg^{-/-}$; $Tie2\text{-}Cre^{-/-}$) was observed at E18.5; however, approximately 40% of $Arg^{-/-}$ mice died within a week of birth (data not shown). Thus, loss of endothelial expression of both Abl and Arg kinases resulted in more frequent and earlier lethality than did loss of Arg alone.

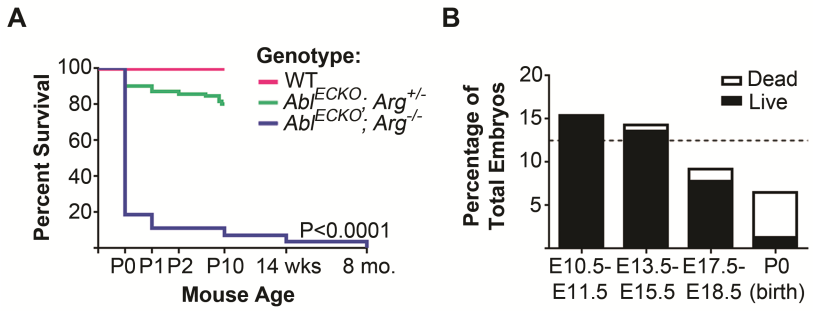

Figure 3.2 Embryonic and Perinatal Lethality of *Abl^{ECKO}; Arg$^{-/-}$* Mice

(**A**) Survival of *Abl^{ECKO}; Arg$^{-/-}$* mice compared to *Abl$^{flox/flox}$; Arg$^{+/+}$; Tie2-Cre$^{-/-}$* (wild-type, WT) and *Abl$^{flox/flox}$; Arg$^{+/-}$; Tie2-Cre$^{+/-}$* (*Abl^{ECKO}; Arg$^{+/-}$*) littermates (P0 = postnatal day 0, birth; n=462 total pups examined). (**B**) Survival of *Abl^{ECKO}; Arg$^{-/-}$* (mutant) embryos at various stages of gestation, relative to expected Mendelian frequency (12.5%, indicated by dashed line).

Surviving late-stage *Abl^{ECKO}; Arg$^{-/-}$* embryos were indistinguishable from wild-type (*Abl$^{flox/flox}$; Arg$^{+/+}$; Tie2-Cre$^{-/-}$*) littermates, with no defects in gross vascular morphology (**Figure 3.3A**). In addition, no changes in overall vascular patterning were observed in the heart or lungs (**Figure 3.3B,C**), nor was smooth muscle cell coverage of vessels affected (**Figure 3.3D**). However, over 50% (42 of 77) of *Abl^{ECKO}; Arg$^{-/-}$* embryos displayed focal areas of hepatic necrosis of varying severity, typically localized to the periphery of the lobes (**Figure 3.4A,B**). Co-staining of liver sections for the endothelial marker CD31 (also known as PECAM-1) and apoptosis marker cleaved caspase-3 showed dramatic reduction in vascular density and extensive apoptosis in the peripheral necrotic areas (**Figure 3.4C**), suggesting a loss of vascular perfusion in these regions.

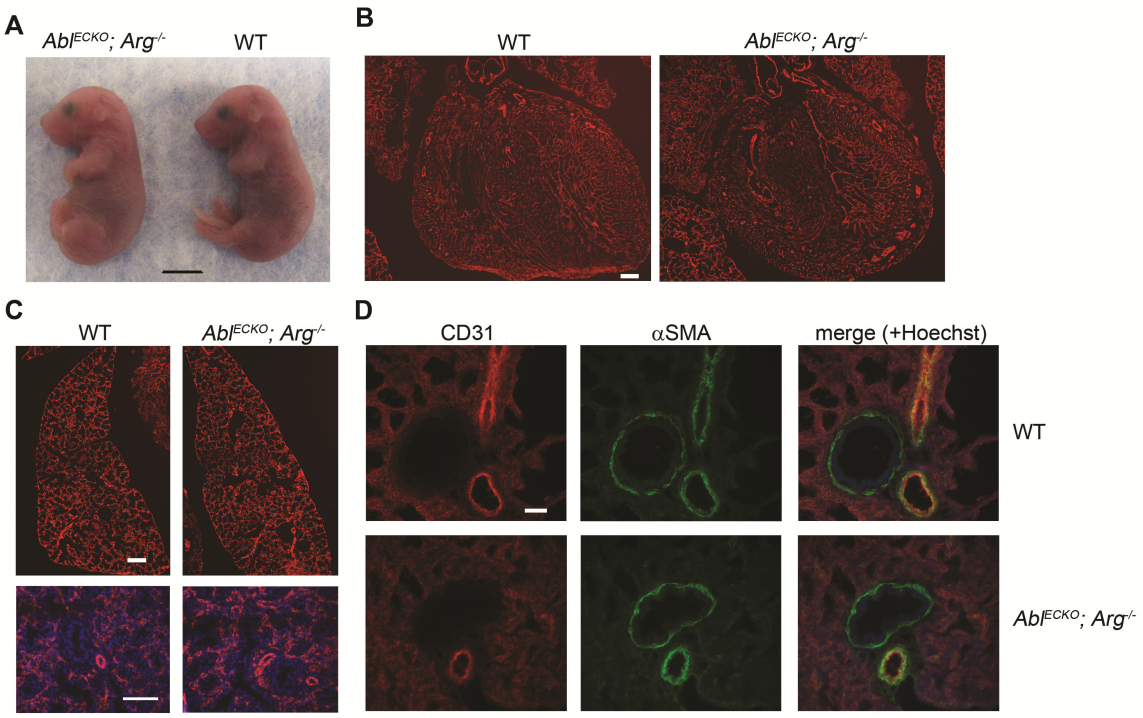

Figure 3.3 Normal Overall Vascular Morphology and Patterning in *Abl^{ECKO}; Arg$^{-/-}$* Embryos

(**A**) Image of E18.5 WT and *Abl^{ECKO}; Arg$^{-/-}$* embryos. Scale bar = 1mm. (**B**) CD31 staining of heart sections from E18.5 WT and *Abl^{ECKO}; Arg$^{-/-}$* embryos. Scale bar = 100μm. (**C**) CD31 staining (red) of lung sections from E18.5 WT and *Abl^{ECKO}; Arg$^{-/-}$* embryos. Scale bars = 100μm (top panels); 50μm (bottom panels). (**D**) Co-staining of lung sections from E18.5 WT and *Abl^{ECKO}; Arg$^{-/-}$* embryos for CD31 (red) and α-smooth muscle actin (αSMA, green). Scale bar = 20μm.

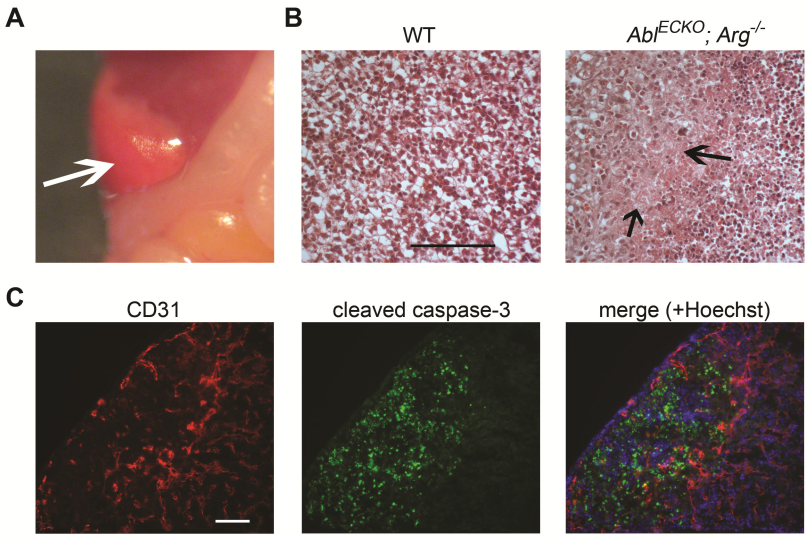

Figure 3.4 Localized Loss of Vasculature and Necrosis/Apoptosis in Livers of *Abl*^{ECKO}; *Arg*$^{-/-}$ Embryos

(**A**) E18.5 mutant liver, displaying peripheral necrosis (arrow). (**B**) Hematoxylin/eosin (H&E)-stained sections of E18.5 WT and mutant livers, demonstrating necrotic areas in mutant liver (arrows). Scale bar = 100μm. (**C**) E18.5 mutant liver section co-stained for CD31/PECAM-1 (endothelial cell marker, red) and cleaved caspase-3 (apoptosis marker, green). Scale bar = 50μm.

As *Tie2-Cre* also drives *Abl* inactivation in hematopoietic cells [363], we additionally examined hematopoietic progenitor cells in mutant fetal livers. No differences in percentages of lineage-negative (Lin^-)/Kit^+ hematopoietic progenitors or erythroid marker TER-119/CD71 double-positive progenitors were observed in mutant embryos compared to wild-type littermates (**Table 3.1**). Thus, together our data suggest that loss of Abl kinases in endothelial cells results in lethality linked to loss of vascular function late in development.

Table 3.1 No Differences in Hematopoietic Progenitors in *Abl^{ECKO}; Arg$^{-/-}$* Embryos

Cell Marker	WT	*Abl^{ECKO}; Arg$^{-/-}$*
% TER-119^{+}	80.54 +/- 2.58	81.07 +/- 4.15
% CD71^{+}	92.48 +/- 2.50	92.93 +/- 3.35
% TER-119^{+}/CD71^{+}	79.34 +/- 3.07	80.23 +/- 4.61
% Lin^{-}	20.70 +/- 1.98	20.80 +/- 2.92
% Kit^{+}	14.40 +/- 2.02	14.13 +/- 2.58
% Lin^{-}/Kit^{+}	12.66 +/- 1.04	12.60 +/- 1.70

Fetal liver cells were isolated from E15.5 wild-type (WT) and *Abl^{ECKO}; Arg$^{-/-}$* embryos and their expression of various hematopoietic markers assessed by flow cytometry. No differences in the percentages of lineage-negative (Lin^{-})/Kit^{+} hematopoietic progenitors or TER-119^{+}/CD71^{+} erythroid progenitors were observed. Data are presented as means (%) +/- SD (WT, n=5 embryos; *Abl^{ECKO}; Arg$^{-/-}$*, n=3 embryos).

3.2.2 Cardiac Enlargement and Scarring in *Abl^{ECKO}; Arg$^{+/-}$* Mice

To examine the role of endothelial Abl kinases in vascular structure and function in adult mice, we employed endothelial *Abl*-deficient mice on an *Arg$^{+/-}$* background (*Abl$^{flox/flox}$; Arg$^{+/-}$; Tie2-Cre$^{+/-}$*, hereafter referred to as *Abl^{ECKO}; Arg$^{+/-}$*), which survive to adulthood. The majority of adult *Abl^{ECKO}; Arg$^{+/-}$* mice did not display any obvious cardiovascular pathology, although a slight decrease in left ventricular function (% fractional shortening) was observed (**Table 3.2**).

Table 3.2 Echocardiographic Evaluation of Left Ventricular Function in Adult *Abl*^{ECKO}; *Arg*$^{+/-}$ Mice

Echocardiographic Analysis Parameter	***Arg*$^{+/-}$ control** (n=30)	***Abl*^{ECKO}; *Arg*$^{+/-}$** (n=30)
FS (%)	56.34 +/- 3.48	53.93 +/- 4.40 *
LVDd (mm)	2.93 +/- 0.27	2.97 +/- 0.31
LVDs (mm)	1.28 +/- 0.18	1.37 +/- 0.21
IVSW (mm)	1.02 +/- 0.10	0.98 +/- 0.12
PW (mm)	1.00 +/- 0.12	0.98 +/- 0.11
LVm (mg)	103.3 +/- 19.7	100.3 +/- 18.7
ET (ms)	39.15 +/- 2.71	38.06 +/- 3.06
HR (bpm)	671.7 +/- 50.0	666.3 +/- 41.8
mVcf (circ/s)	14.47 +/- 1.40	14.22 +/- 1.25
mVcfc (circ/s)	4.32 +/- 0.32	4.27 +/- 0.37

Left ventricular function of 4- to 5-month-old female *Abl*^{ECKO}; *Arg*$^{+/-}$ mice and age/sex-matched *Arg*$^{+/-}$ control mice (*Abl*$^{flox/flox}$; *Arg*$^{+/-}$; *Tie2-Cre*$^{-/-}$) was evaluated by echocardiography (unanesthetized mice). These studies were performed by Lan Mao in Howard Rockman's laboratory (Departments of Medicine, Cell Biology, and Molecular Genetics; Duke University School of Medicine). Data are shown as means +/- SD. (*P<0.05). Abbreviations: FS, fractional shortening; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; IVSW, interventricular septum width (thickness); PW, posterior wall width (thickness); LVm, left ventricular mass; ET, ejection time; HR, heart rate; bpm, beats per minute; mVcf, mean velocity of circumferential fiber shortening; circ/s, circumferential shortening per second; mVcfc, heart rate-corrected mVcf.

However, approximately 15% (17 of 118) of the *Abl*^{ECKO}; *Arg*$^{+/-}$ mice were severely runted (body weights less than 75% of *Abl*$^{flox/flox}$; *Arg*$^{+/-}$; *Tie2-Cre*$^{-/-}$ littermate controls) and displayed dramatic cardiovascular phenotypes by 2-3 months of age. The hearts of these mice were enlarged, typically with prominent dilation of the left atrium (**Figure 3.5A,B**). Histological analysis of cardiac tissue sections demonstrated localized regions of collagen deposition and scarring in the left ventricle (**Figure 3.5C**).

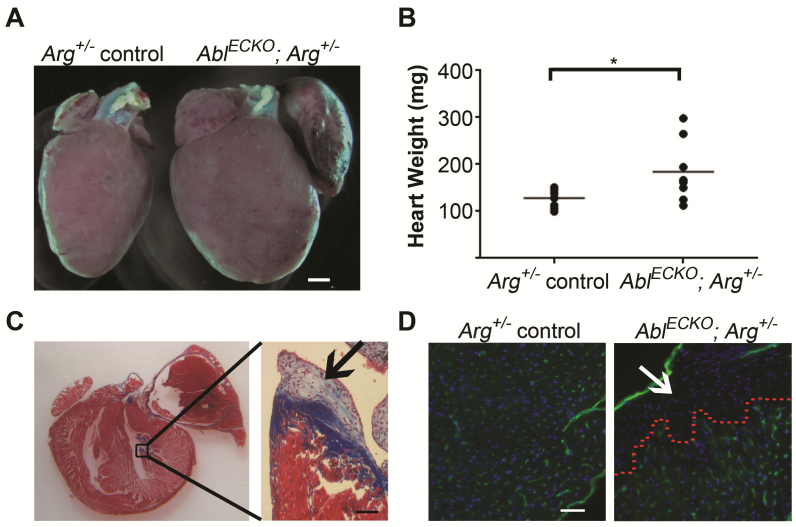

Figure 3.5 Cardiac Enlargement and Scarring in *Abl*^{ECKO}; *Arg*$^{+/-}$ Mice

(**A)** Stereoscopic image of hearts from 8-week-old *Arg*$^{+/-}$ control (*Abl*$^{flox/flox}$; *Arg*$^{+/-}$; *Tie2-Cre*$^{-/-}$) and *Abl*^{ECKO}; *Arg*$^{+/-}$ mice, demonstrating overall heart enlargement and dilated left atrium in *Abl*^{ECKO}; *Arg*$^{+/-}$ mouse. Scale bar = 1mm. (**B**) Quantification of weights of hearts from *Arg*$^{+/-}$ control and *Abl*^{ECKO}; *Arg*$^{+/-}$ mice (lines indicate mean values, n=8 mice/genotype; *P<0.05). (**C**) Trichrome staining of *Abl*^{ECKO}; *Arg*$^{+/-}$ heart, displaying scarring in the left ventricle (magnified in inset, arrow). Scale bar = 50μm. (**D**) CD31 staining (green) of *Abl*^{ECKO}; *Arg*$^{+/-}$ left ventricle. A complete loss of capillaries was observed in the scarred region (above red dotted line, arrow). Scale bar = 20μm.

While overall capillary density in the heart and skeletal muscle of *Abl*^{ECKO}; *Arg*$^{+/-}$ mice was not significantly different from *Arg*$^{+/-}$ controls (**Figure 3.6A,B**), a near-complete loss of blood vessels was observed in the scarred regions (**Figure 3.5D**). These findings are consistent with the localized loss of vasculature observed in the livers of *Abl*^{ECKO}; *Arg*$^{-/-}$ embryos (**Figure 3.4C**) and suggest a critical role for the Abl kinases in vascular maintenance and function. Interestingly, these *Abl*^{ECKO}; *Arg*$^{+/-}$ mice also displayed thickening of the right ventricular wall, which correlated with cardiomyocyte hypertrophy (**Figure 3.7A,B**).

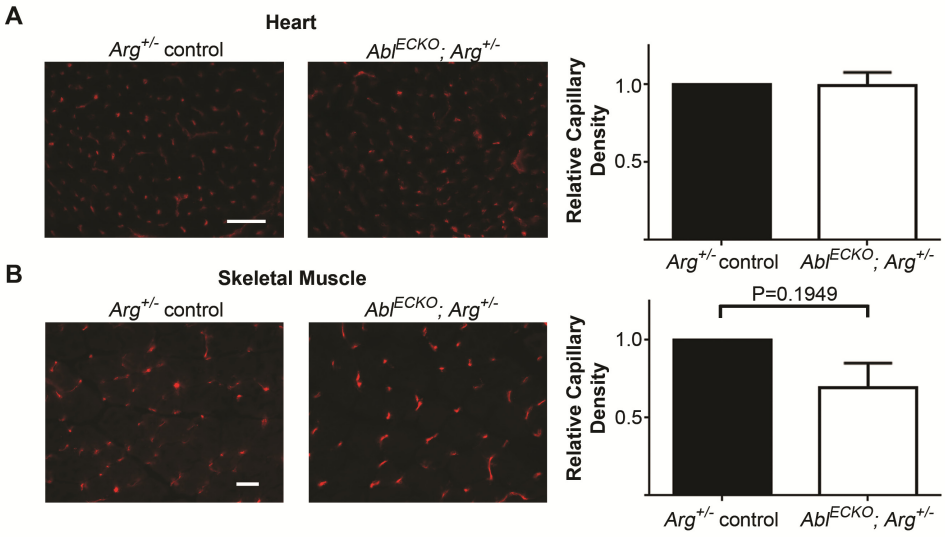

Figure 3.6 Normal Capillary Density in Heart and Skeletal Muscle of *Abl*^{ECKO}; *Arg*$^{+/-}$ Adult Mice

(**A**) CD31 staining of heart cross-sections from 8-week-old *Arg*$^{+/-}$ control (*Abl*$^{flox/flox}$; *Arg*$^{+/-}$; *Tie2-Cre*$^{-/-}$) and *Abl*^{ECKO}; *Arg*$^{+/-}$ mice, revealing comparable capillary density, quantified in right panel (means +/- SD, relative to capillary density in *Arg*$^{+/-}$ control hearts, n=3 mice/genotype). (**B**) CD31 staining of tibialis anterior muscle cross-sections from 8-week-old *Arg*$^{+/-}$ control and *Abl*^{ECKO}; *Arg*$^{+/-}$ mice. No significant difference was observed, although a trend toward decreased capillary density was noted in *Abl*^{ECKO}; *Arg*$^{+/-}$ mice

(right panel; means +/- SD, relative to capillary density in $Arg^{+/-}$ control muscle, n=3 mice/genotype). Scale bars in (**A**) and (**B**) = 20μm.

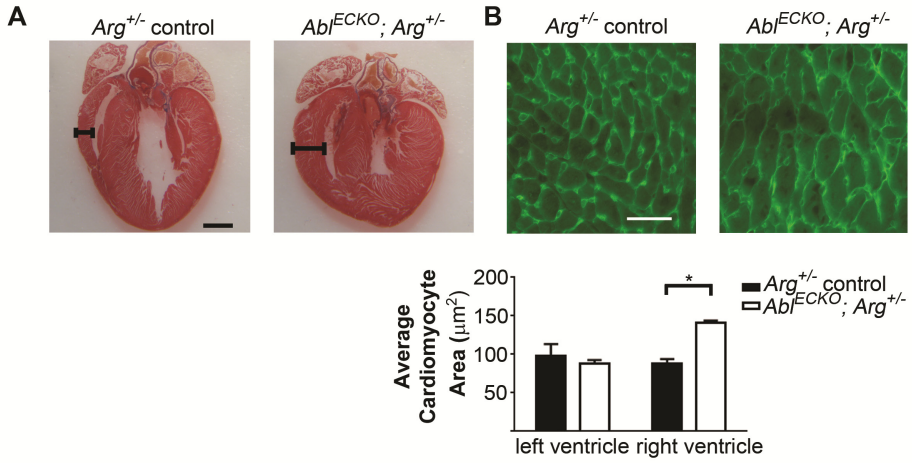

Figure 3.7 Right Ventricular Hypertrophy in Abl^{ECKO}; $Arg^{+/-}$ Mice

(**A**) Trichrome-stained heart sections from 8-week-old $Arg^{+/-}$ control ($Abl^{flox/flox}$; $Arg^{+/-}$; *Tie2-Cre*$^{-/-}$) and Abl^{ECKO}; $Arg^{+/-}$ mice, displaying increased right ventricle wall thickness in Abl^{ECKO}; $Arg^{+/-}$ heart (brackets). Scale bar = 1mm. (**B**) FITC-wheat germ agglutinin staining of right ventricle cross-sections, demonstrating increased cardiomyocyte cross-sectional area in Abl^{ECKO}; $Arg^{+/-}$ right ventricle, quantified in bottom panel (means +/- SD, n=3 mice/genotype; *P<0.05). Scale bar = 20μm.

3.2.3 Lung Fibrosis and Thrombosis in Abl^{ECKO}; $Arg^{+/-}$ Mice

As right ventricular hypertrophy may occur as a consequence of altered pulmonary circulation and hypertension [364], we examined the lungs of Abl^{ECKO}; $Arg^{+/-}$ mice. The lungs of these mice were enlarged and dense, with prominent white, fibrous areas (**Figure 3.8A,B**). Histological analysis of pulmonary structure showed extensive interstitial fibrosis (**Figure 3.8C**), along with fibrin deposition in the airways (**Figure 3.8 D,E**), indicative of prior hemorrhage and defective pulmonary vascular integrity. We also observed a dramatic loss of vascular density, as assessed by staining for the

endothelial marker von Willebrand factor (vWF) (**Figure 3.8E)**. An abundance of hemosiderin-laden macrophages was detected in the lungs of *Abl*^{ECKO}*; Arg*$^{+/-}$ mice (**Figure 3.8F**); the presence of these cells has been associated with heart failure [365]. Thus, defective left ventricular function may contribute to the observed lung abnormalities, by producing congestion of the lung vasculature, with resulting leakage of red blood cells.

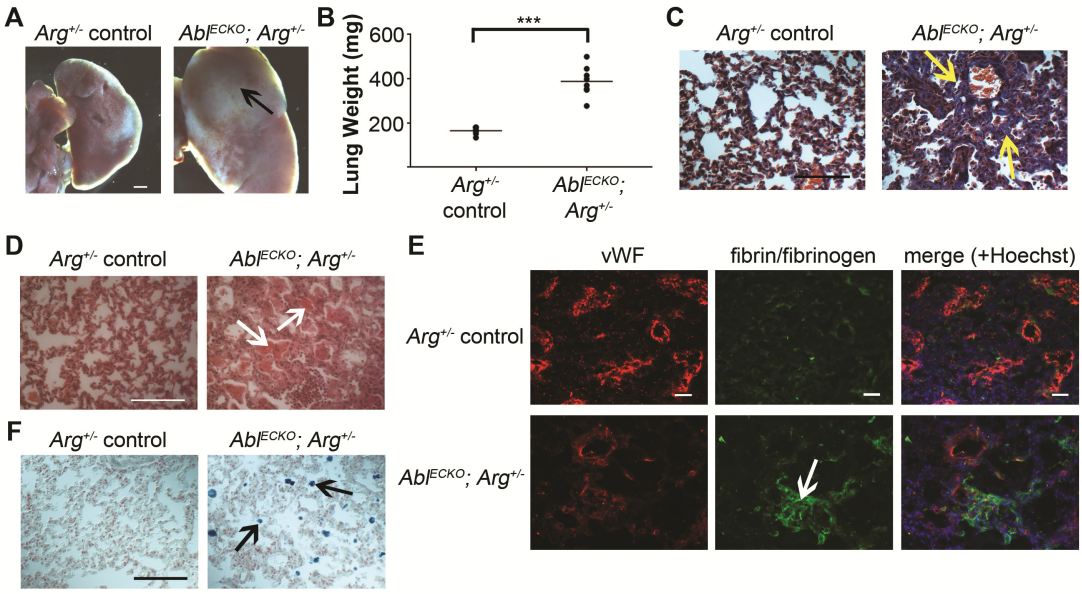

Figure 3.8 Pulmonary Fibrosis in *Abl*^{ECKO}*; Arg*$^{+/-}$ Mice

(**A**) Stereoscope image of lungs from 8-week-old *Arg*$^{+/-}$ control and *Abl*^{ECKO}*; Arg*$^{+/-}$ mice. *Abl*^{ECKO}*; Arg*$^{+/-}$ lungs displayed regions of apparent fibrosis (arrow). Scale bar = 1mm. (**B**) Quantification of weights of lungs from *Arg*$^{+/-}$ control and *Abl*^{ECKO}*; Arg*$^{+/-}$ mice (lines indicate mean values, n=8 mice/genotype; ***P<0.001). (**C**) Trichrome staining, displaying collagen deposition in *Abl*^{ECKO}*; Arg*$^{+/-}$ lung (blue staining; arrows). Scale bar = 100μm. (**D**) H&E staining of lung sections, demonstrating fibrin deposition in airways of *Abl*^{ECKO}*; Arg*$^{+/-}$ lung (arrows). Scale bar = 100μm. (**E**) Co-staining of lung sections for vWF (endothelial cell marker, red) and fibrin/fibrinogen (green). Loss of vessel density, as well as fibrin deposition (arrow), was observed in *Abl*^{ECKO}*; Arg*$^{+/-}$ lung. Scale bars = 20μm. (**F**) Prussian blue (iron) staining, showing accumulation of hemosiderin-laden macrophages (blue staining; arrows) in *Abl*^{ECKO}*; Arg*$^{+/-}$ lung. Scale bar = 100μm.

Interestingly, no overt cardiac and pulmonary phenotypes were observed in E18.5 *Abl*^{ECKO}; *Arg*$^{-/-}$ embryos (**Figure 3.9A,B**), suggesting that these defects develop later in adult *Abl*^{ECKO}; *Arg*$^{+/-}$ mice, potentially as a result of cumulative injury or stress in the adult vasculature. Importantly, Arg protein levels were comparable in *Arg*$^{+/-}$ control and *Abl*^{ECKO}; *Arg*$^{+/-}$ mice (**Figure 3.10 A,B**), and no cardiac hypertrophy or pulmonary fibrosis was observed in adult *Arg*$^{-/-}$ mice (**Figure 3.11A-E**), suggesting that the observed cardiovascular phenotypes result from endothelial Abl, rather than Arg, depletion in *Abl*^{ECKO}; *Arg*$^{+/-}$ adult mice.

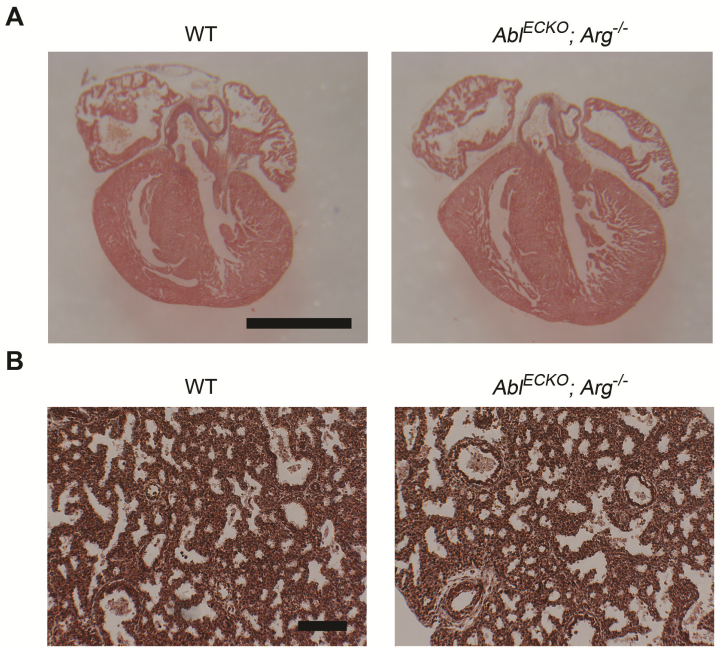

Figure 3.9 Lack of Cardiac Abnormalities and Pulmonary Fibrosis in *Abl*^{ECKO}; *Arg*$^{-/-}$ Embryos

(**A**) H&E-stained heart sections from E18.5 *Abl*$^{flox/flox}$; *Arg*$^{+/+}$; *Tie2-Cre*$^{-/-}$ (wild-type, WT) and *Abl*^{ECKO}; *Arg*$^{-/-}$ embryos. Scale bar = 1mm. (**B**) H&E-stained lung sections from E18.5 wild-type (WT) and *Abl*^{ECKO}; *Arg*$^{-/-}$ embryos. Scale bar = 100μm.

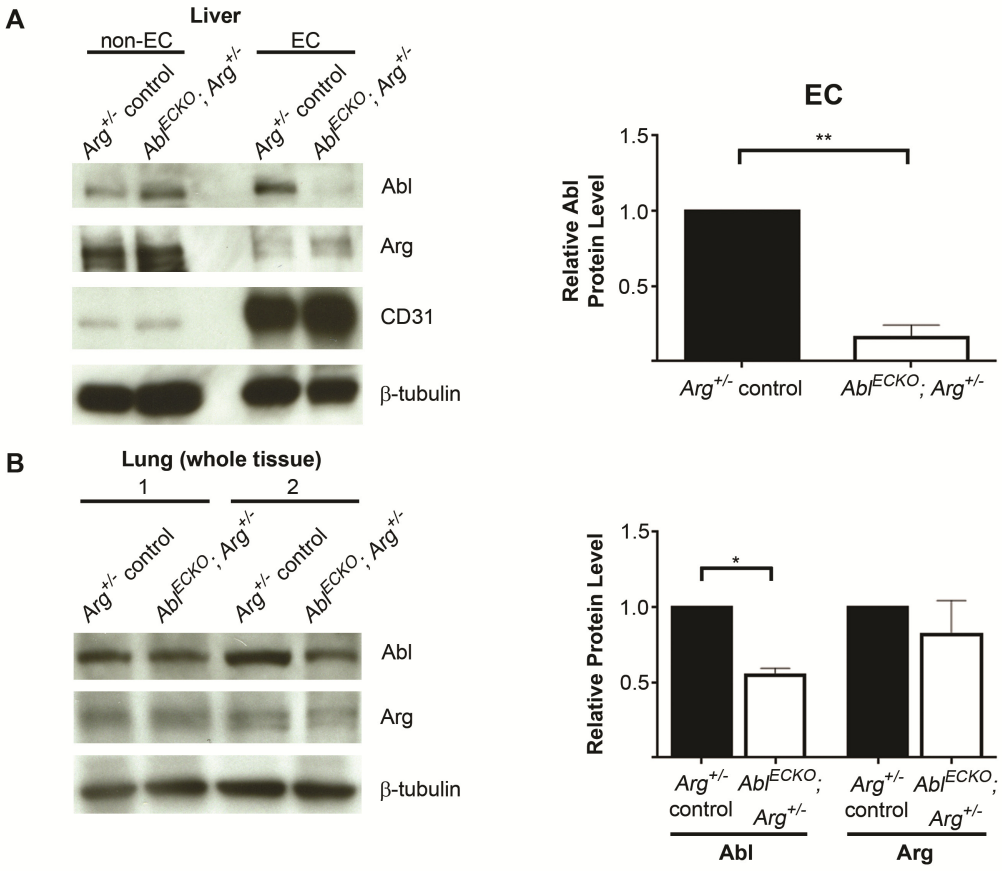

Figure 3.10 Characterization of Abl and Arg Protein Levels in *Abl^{ECKO}; Arg$^{+/-}$* Adult Mice

(**A**) Analysis of Abl and Arg protein levels in endothelial (EC; CD105^{+}/CD45^{-}/CD31^{+}) and non-hematopoietic/non-endothelial (non-EC; CD105^{-}/CD45^{-}/CD31^{-}) liver cell populations from *Arg$^{+/-}$* control (*Abl$^{flox/flox}$; Arg$^{+/-}$; Tie2-Cre$^{-/-}$*) and *Abl^{ECKO}; Arg$^{+/-}$* adult mice, demonstrating endothelial cell-specific Abl depletion in *Abl^{ECKO}; Arg$^{+/-}$* mice, quantified in right panel (means +/- SD, n=3). (**B**) Analysis of Abl and Arg protein levels in whole tissue homogenates from lungs of two pairs of *Arg$^{+/-}$* control and *Abl^{ECKO}; Arg$^{+/-}$* adult mice, quantified in right panel (means +/- SD, n=3). While Arg protein levels were comparable in whole tissue homogenates from lungs of *Arg$^{+/-}$* control and *Abl^{ECKO}; Arg$^{+/-}$* mice, reduced Abl levels were observed in *Abl^{ECKO}; Arg$^{+/-}$* lungs, likely as a result of the high vascular content of this tissue. (*P<0.05; **P<0.01).

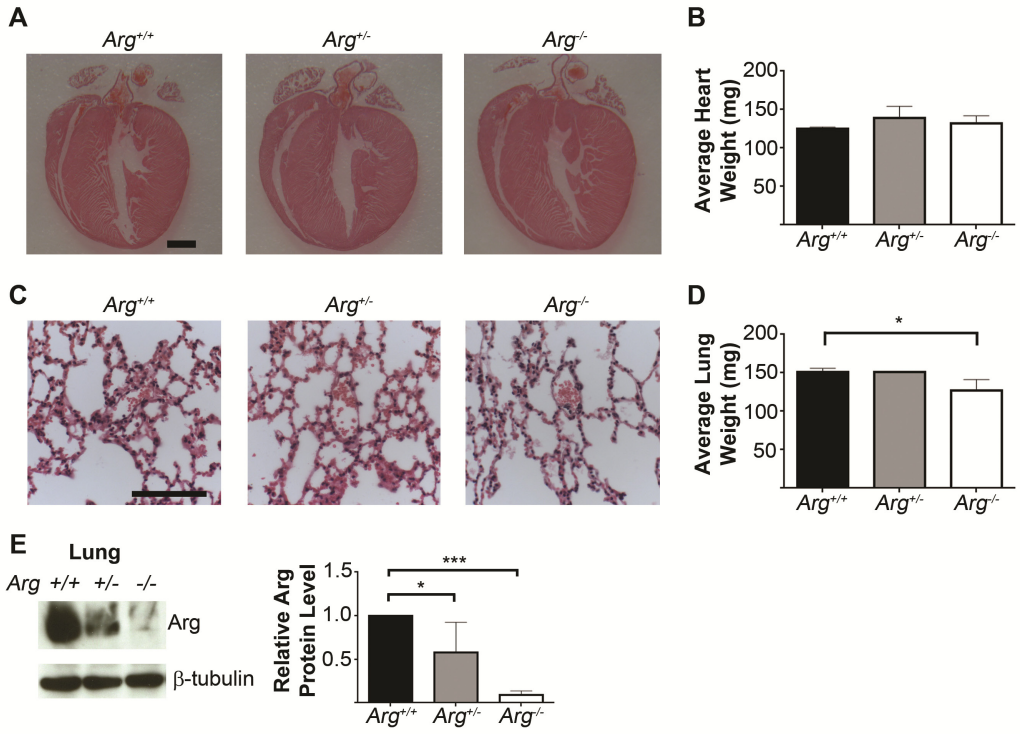

Figure 3.11 Lack of Cardiac Hypertrophy and Pulmonary Fibrosis in *Arg*$^{-/-}$ Adult Mice

(**A**) H&E-stained heart sections from 8-week-old *Arg*$^{+/+}$ (*Abl*$^{flox/flox}$; *Arg*$^{+/+}$; *Tie2-Cre*$^{-/-}$), *Arg*$^{+/-}$ (*Abl*$^{flox/flox}$; *Arg*$^{+/-}$; *Tie2-Cre*$^{-/-}$), and *Arg*$^{-/-}$ (*Abl*$^{flox/flox}$; *Arg*$^{-/-}$; *Tie2-Cre*$^{-/-}$) mice. Scale bar = 1mm. (**B**) Quantification of weights of hearts from *Arg*$^{+/+}$, *Arg*$^{+/-}$, and *Arg*$^{-/-}$ adult mice (means +/- SD, n=3 mice/genotype). (**C**) H&E-stained lung sections from 8-week old *Arg*$^{+/+}$, *Arg*$^{+/-}$, and *Arg*$^{-/-}$ mice. Scale bar = 100μm. (**D**) Quantification of weights of lungs from *Arg*$^{+/+}$, *Arg*$^{+/-}$, and *Arg*$^{-/-}$ adult mice (means +/- SD, n=3 mice/genotype). A mild reduction in average lung weight was noted in *Arg*$^{-/-}$ mice, likely resulting from the slightly lower body weights of these animals (body weights approximately 85-90% of wild-type littermates). (**E**) Assessment of Arg protein levels in lung homogenates from *Arg*$^{+/+}$, *Arg*$^{+/-}$, and *Arg*$^{-/-}$ adult mice, quantified in right panel (means +/- SD, n=3 mice/genotype). (*P<0.05; ***P<0.01).

The occurrence of liver necrosis in *Abl*^{ECKO}; *Arg*$^{-/-}$ embryos and left ventricular scarring in adult *Abl*^{ECKO}; *Arg*$^{+/-}$ mice suggests that loss of endothelial Abl kinases results in localized defects in tissue perfusion, with infarctions leading to tissue death and

scarring. In this regard, we observed sporadic thrombi in lung and liver microvessels of *Abl*^{ECKO}*; Arg*$^{+/-}$ adult mice, which were not seen in vessels of *Arg*$^{+/-}$ control mice. These thrombi stained positively for vWF, fibrin (**Figure 3.12A**), and the platelet fibrinogen receptor integrin αIIb (CD41) (**Figure 3.12B**). Importantly, these thrombi were observed in mice without any outward cardiac pathology, suggesting that their occurrence is not a secondary effect of compromised cardiac function. Histological analysis revealed abnormally swollen endothelial cells adjacent to a lung thrombus (**Figure 3.12C**, *right panel*), consistent with endothelial injury contributing to thrombosis. These findings suggest that loss of Abl kinases perturbs vascular function as a result of endothelial cell damage or death.

3.2.4 Increased Apoptosis Following Loss of Endothelial Abl Kinases

Global *Abl*/*Arg* knockout mice displayed increased apoptosis in all tissues [329], suggesting that the Abl kinases may have an important pro-survival function. Thus, we examined whether loss of Abl kinases affected endothelial cell viability *in vivo*. Indeed, a significant increase in cleaved caspase-3-positive cells, as well as numerous CD31/cleaved caspase-3 double-positive endothelial cells, were observed in *Abl*^{ECKO}*; Arg*$^{-/-}$ embryo lungs (**Figure 3.13A-C**), demonstrating that loss of endothelial Abl kinases led to increased apoptosis. This finding is consistent with the extensive apoptosis detected in *Abl*^{ECKO}*; Arg*$^{-/-}$ embryo livers (**Figure 3.4C**).

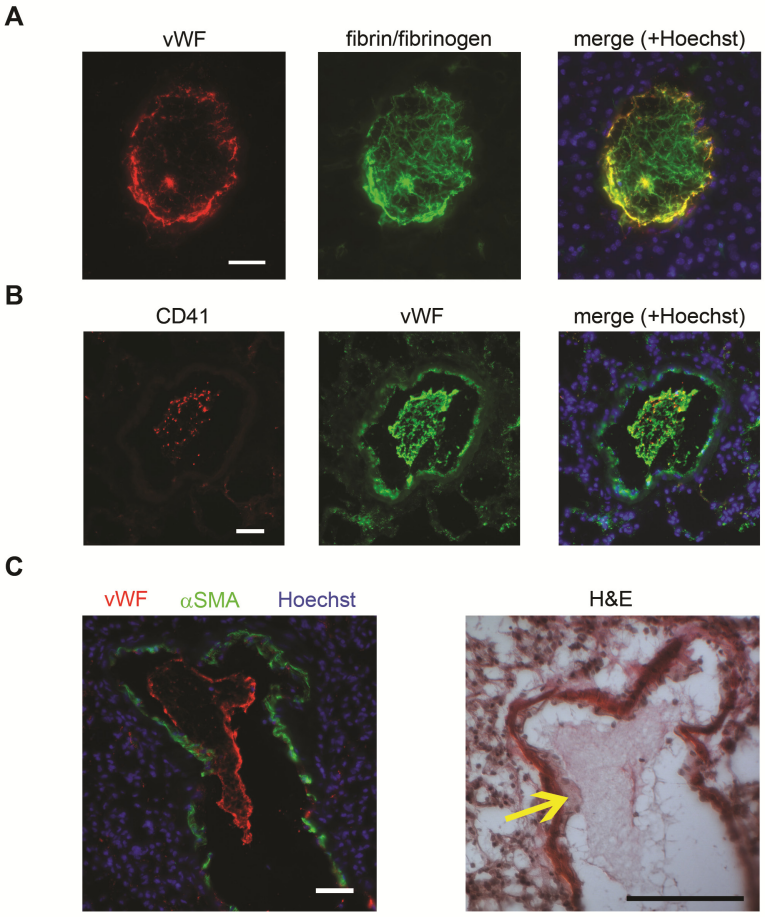

Figure 3.12 Vascular Occlusions in *Abl*^{ECKO}; *Arg*$^{+/-}$ Mice

(**A**) Co-staining of *Abl*^{ECKO}; *Arg*$^{+/-}$ liver sections for vWF (red) and fibrin/fibrinogen (green). (**B**) Co-staining of *Abl*^{ECKO}; *Arg*$^{+/-}$ lung sections for CD41 (platelet marker, red) and vWF (green). Scale bars in (**A**) and (**B**) = 20μm. (**C**) *Left panel*: Co-staining of *Abl*^{ECKO}; *Arg*$^{+/-}$ lung section for vWF (red) and α-smooth muscle actin (αSMA, green). Scale bar = 20μm. *Right panel*: H&E staining of *Abl*^{ECKO}; *Arg*$^{+/-}$ lung section, demonstrating abnormal endothelial morphology at site of lung thrombus (arrow). Scale bar = 100μm.

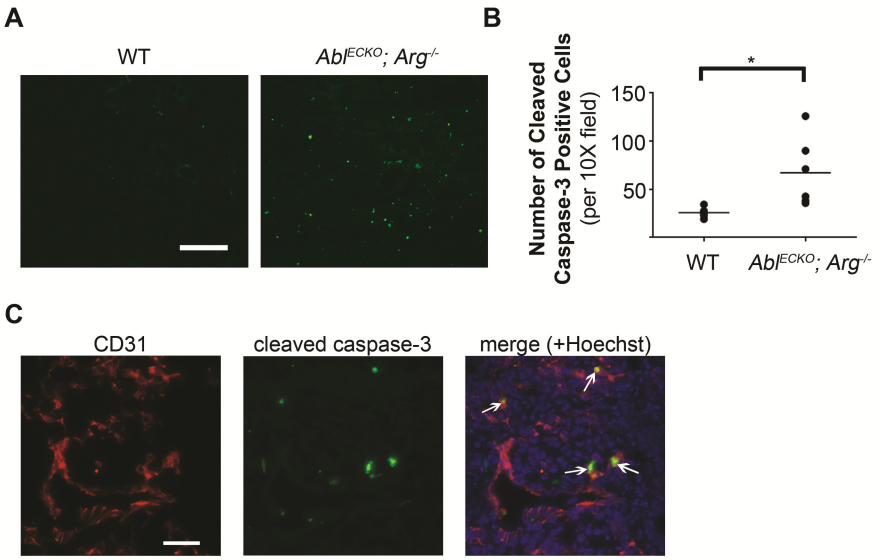

Figure 3.13 Increased Apoptosis Following Loss of Endothelial Abl Kinases *In Vivo*

(**A** and **B**) Staining of lung sections from E18.5 *Abl*$^{flox/flox}$; *Arg*$^{+/+}$; *Tie2-Cre*$^{-/-}$ (wild-type, WT) and *Abl*^{ECKO}; *Arg*$^{-/-}$ (mutant) embryos for cleaved caspase-3 (green), revealing an increased number of apoptotic cells in mutant lungs, quantified in (**B**) (lines indicate mean values, n=6 embryos/genotype; *P<0.05). Scale bar = 100μm. (**C**) Co-staining of E18.5 mutant lungs for CD31 (red) and cleaved caspase-3 (green), demonstrating double-positive apoptotic endothelial cells (arrows). Scale bar = 20μm.

To confirm that the observed endothelial cell apoptosis represented a cell-autonomous phenotype, we examined the role of the Abl kinases in endothelial cell survival *in vitro*. Treatment of primary human umbilical vein endothelial cells (HUVECs) with the Abl pharmacological inhibitor imatinib decreased cell viability and increased levels of apoptosis following serum-starvation (**Figure 3.14A,B**). The pro-survival effects of both VEGF and basic fibroblast growth factor (bFGF) also were decreased in the presence of the Abl inhibitor. Treatment with either VEGF or bFGF led

to increased Abl kinase activation, as assessed by the phosphorylation of CrkL at Y207, an Abl-specific phosphorylation site [366] (**Figure 3.14C,D**), suggesting that the Abl kinases might modulate pro-survival signaling downstream of the VEGF and bFGF receptors. Interestingly, imatinib treatment did not increase apoptosis in HUVECs maintained in serum-containing medium (**Figure 3.14 B**), suggesting that the Abl kinases may support survival specifically under stress conditions such as nutrient deprivation, as well as downstream of pro-angiogenic factors. Similarly, microRNA (miRNA)-mediated *Abl*/*Arg* knockdown in HUVECs led to increased apoptosis in response to serum-starvation stress (**Figure 3.14 E,F**). These findings demonstrate an important pro-survival role for the Abl kinases in endothelial cells.

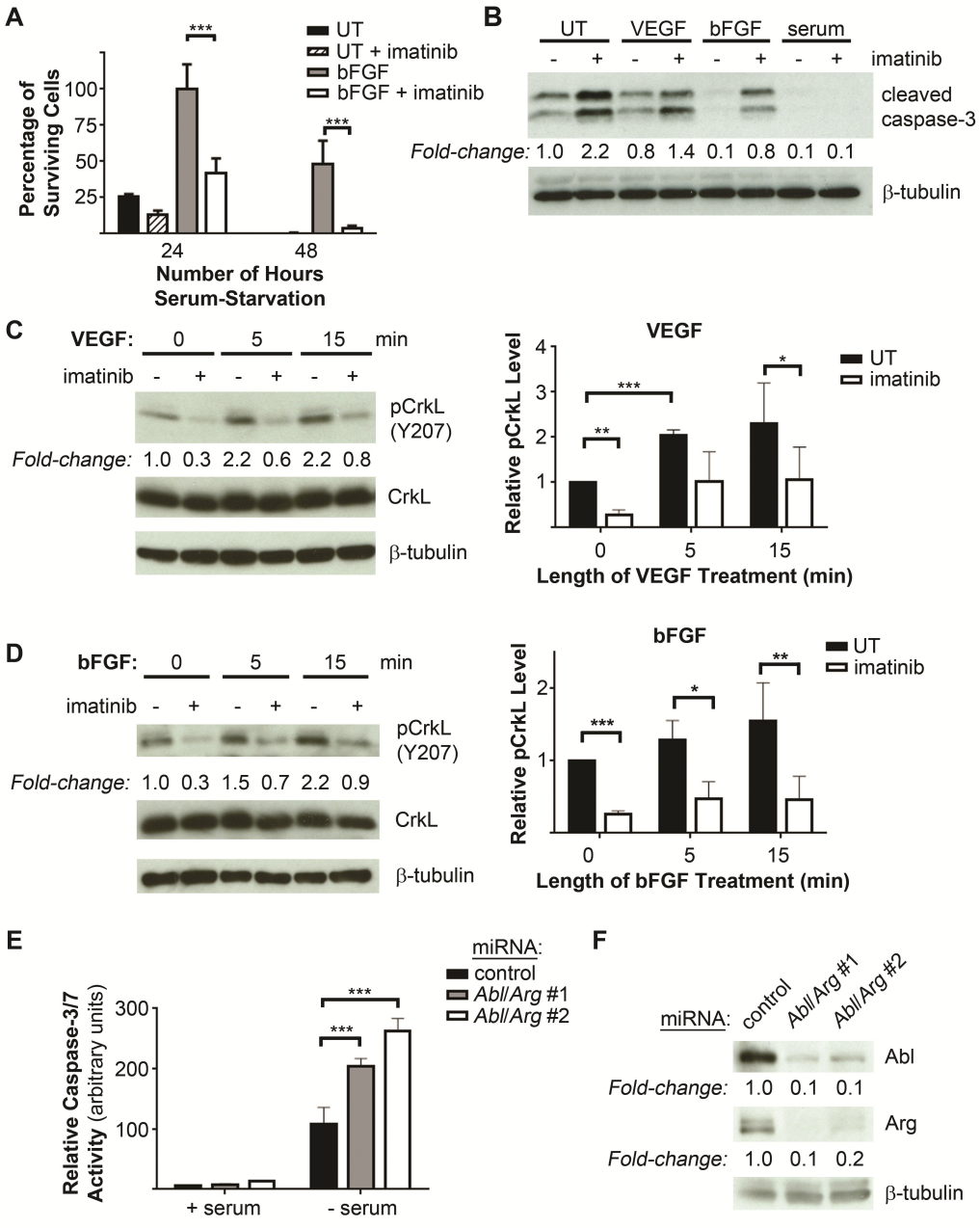

Figure 3.14 Increased Apoptosis Following Loss of Endothelial Abl Kinases *In Vitro*

(**A**) Viability of primary HUVECs serum-starved (UT) or supplemented with bFGF (10ng/mL) in serum-free medium, +/- imatinib (10μM), as assessed by MTS assay. Values are expressed relative to viability of bFGF-treated cells 24 hours after serum-starvation. Data are presented as means +/- SD (n=3; ***P<0.001). (**B**) Analysis of cleaved caspase-3

levels (apoptosis) in HUVECs serum-starved and either left untreated (UT) or supplemented with VEGF (100ng/mL) or bFGF (10ng/mL), or maintained in serum-containing medium (serum), +/- imatinib. (**C** and **D**) Assessment of Abl kinase activation, as determined by phospho-CrkL(Y207) levels, following stimulation of serum-starved HUVECs with either (**C**) VEGF or (**D**) bFGF (each 10ng/mL, 5 or 15 minutes) +/- imatinib. Results are quantified in the bottom panels; data are presented as means +/- SD (n=3; *P<0.05; **P<0.01; ***P<0.001). (**E**) Analysis of caspase-3/7 activity in HUVECs expressing control or *Abl*/*Arg* miRNAs either maintained in serum-containing medium (+serum) or serum-starved (-serum) 24 hours. Data are presented as means +/- SD (n=3; ***P<0.001). (**F**) Assessment of Abl/Arg knockdown upon miRNA expression in HUVECs.

3.2.5 Abl Kinases Regulate Tie2 Expression

To determine the pathways whereby Abl kinases impact endothelial cell survival, we examined gene expression differences in HUVECs following *Abl*/*Arg* knockdown, using a real-time RT-PCR array. While mRNA expression of most of the endothelial receptors analyzed was unchanged, *Abl*/*Arg* knockdown led to a greater than 2-fold reduction in *Tie2* (also known as *Tek*) mRNA levels (**Figure 3.15A**). Given the important role of Angpt1/Tie2 signaling in mediating endothelial cell survival and vascular integrity [160,172], we examined the effects of Abl/Arg loss of function on this pathway. Using two *Abl*/*Arg* knockdown constructs, we confirmed by real-time RT-PCR (**Figure 3.16A**) and Western blot analyses (**Figure 3.15B,C**) that both Tie2 mRNA and protein levels were markedly decreased following *Abl*/*Arg* knockdown. Interestingly, levels of Angpt2 were increased following loss of Abl/Arg (**Figures 3.15 B,C and 3.16C**), while *Angpt1* mRNA levels were decreased (**Figure 3.16D**). No consistent difference in *Tie1* receptor expression was observed in cells lacking Abl kinases (**Figure 3.16B**).

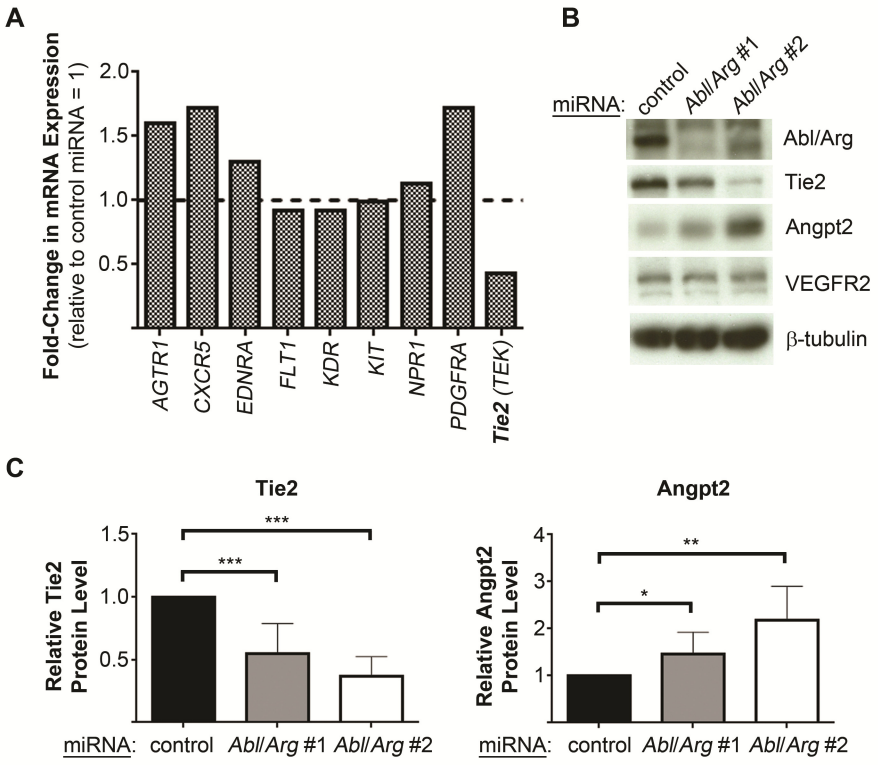

Figure 3.15 Decreased Tie2 Expression Following *Abl*/*Arg* Knockdown

(**A**) Real-time RT-PCR array analysis of gene expression in HUVECs expressing control or *Abl*/*Arg* miRNAs. mRNA expression levels in *Abl*/*Arg*-knockdown HUVECs are shown, relative to levels in cells expressing control miRNA. (*AGTR1* = angiotensin II receptor, type 1; *EDNRA* = endothelin receptor type A; *FLT1* = VEGF receptor 1; *KDR* = VEGF receptor 2; *NPR1* = natriuretic peptide receptor 1). (**B** and **C**) Analysis of Tie2 and angiopoietin-2 (Angpt2) protein levels in HUVECs expressing control miRNA or either of two *Abl*/*Arg* miRNAs, quantified in (**C**) (means +/- SD, n=9; *P<0.05; **P<0.01; ***P<0.001).

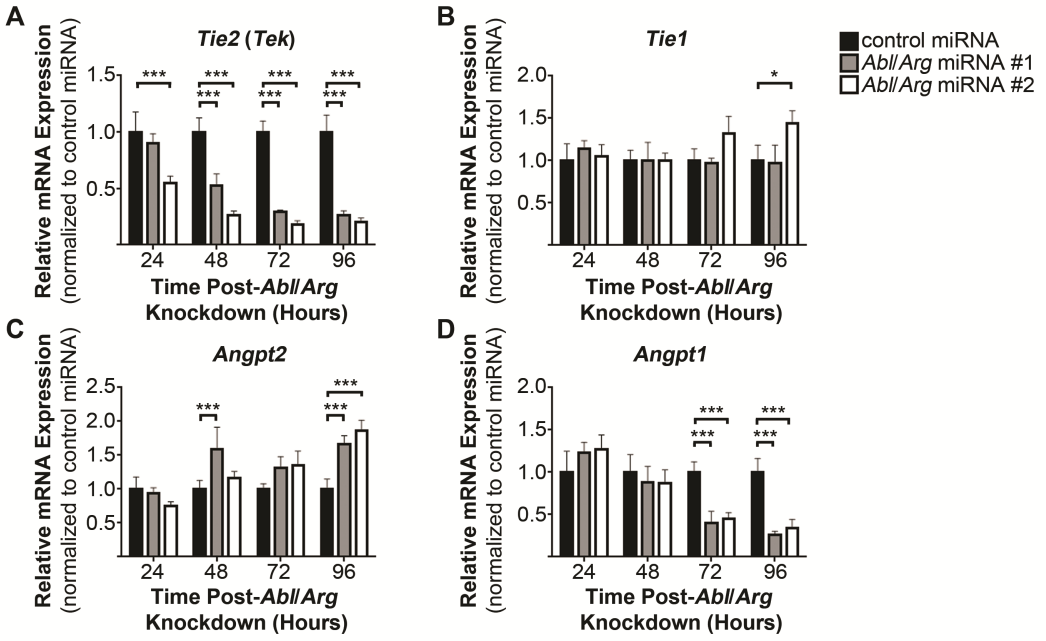

Figure 3.16 Altered Angiopoietin/Tie2 mRNA Levels Following *Abl*/*Arg* Knockdown

Real-time RT-PCR analysis of expression of (**A**) *Tie2* (*Tek*), (**B**) *Tie1*, (**C**) angiopoietin-1 (*Angpt1*), and (**D**) angiopoietin-2 (*Angpt2*) in HUVECs 24 to 96 hours following infection with either control or *Abl*/*Arg* miRNA lentiviruses. Fold-changes in gene expression are shown relative to levels in cells expressing control miRNA. Data are presented as means +/- SD (n=3). (*P<0.05; ***P<0.001).

Importantly, decreased levels of Tie2 protein were also observed in Abl^{ECKO}; $Arg^{-/-}$ embryo liver tissue (**Figure 3.17A**), as well as in liver endothelial cells from E18.5 Abl^{ECKO}; $Arg^{-/-}$ embryos (**Figure 3.17B**), while VEGFR2 levels were unchanged (**Figure 3.17A**). Moreover, a similar decrease in Tie2 expression was observed in primary endothelial cells isolated from $Abl^{flox/flox}$; $Arg^{+/+}$; *Tie2-Cre*$^{-/-}$ mice following *Arg* knockdown and *in vitro* Abl depletion by adenoviral Cre transduction (**Figure 3.17C**).

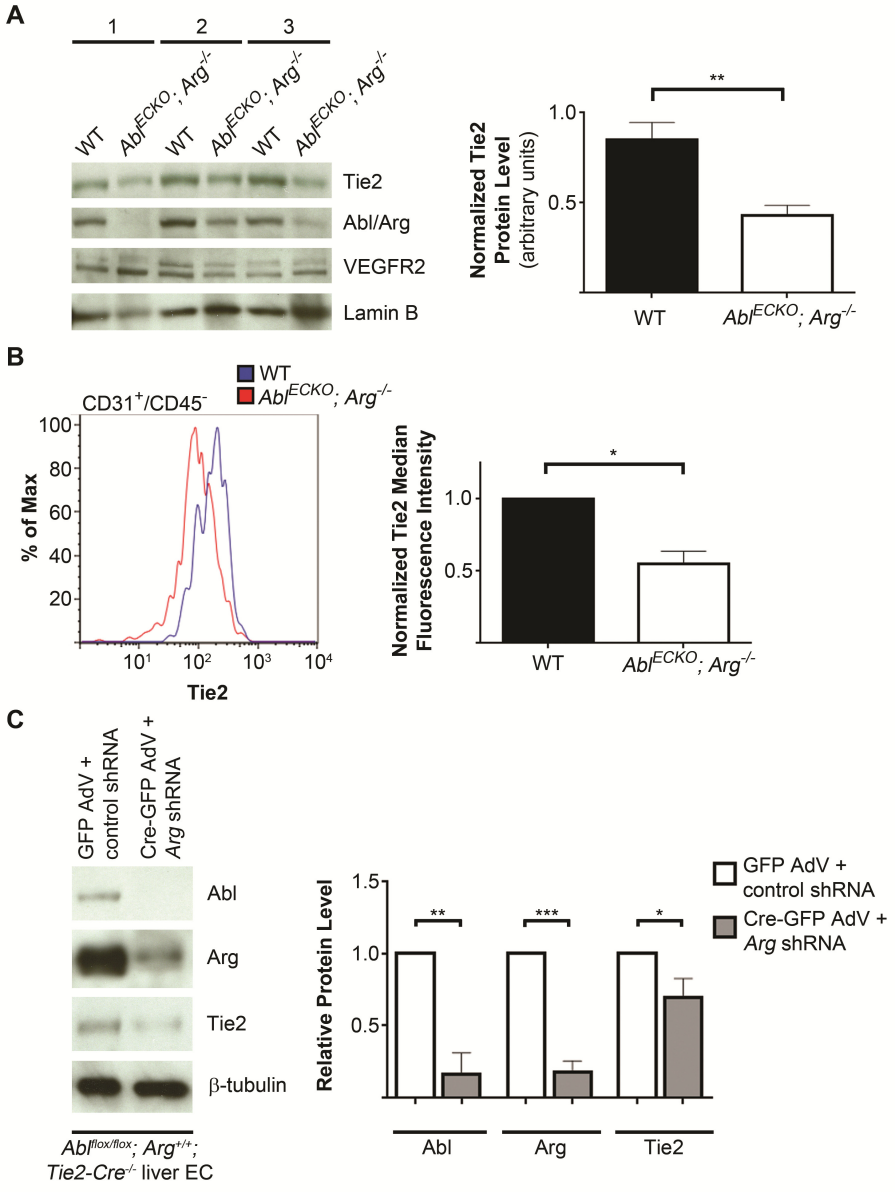

Figure 3.17 Decreased Tie2 Levels Following Loss of Abl/Arg Kinases in Mouse Endothelial Cells

(**A**) Analysis of levels of Tie2 protein in livers from three pairs of *Abl*$^{flox/flox}$; *Arg*$^{+/+}$; *Tie2-Cre*$^{-/-}$ (wild-type, WT) and *Abl*^{ECKO}; *Arg*$^{-/-}$ (mutant) littermate embryos, quantified in right panel (means +/- SD, normalized to Lamin B levels, n=3 mice/genotype; **P<0.01). (**B**) Flow cytometric analysis of Tie2 levels in CD31^{+}/CD45^{-} endothelial cells from E18.5 WT and *Abl*^{ECKO}; *Arg*$^{-/-}$ embryo livers, quantified in right panel (means +/- SD, normalized to

CD31 levels, n=3 mice/genotype; *P<0.05). (**C**) Analysis of levels of Tie2 protein in primary endothelial cells (EC) isolated from livers of *Abl*$^{flox/flox}$; *Arg*$^{+/+}$; *Tie2-Cre*$^{-/-}$ adult mice, following *in vitro* Abl/Arg depletion. Primary liver endothelial cells were infected with either GFP or Cre-GFP-expressing adenoviruses (AdV), followed by lentiviral transduction of either control or *Arg* shRNAs. Abl, Arg, and Tie2 protein levels (normalized to β-tubulin) are quantified in the panel at right, relative to levels in control knockdown cells (means +/- SD, n=3; *P<0.05; **P<0.01; ***P<0.001).

3.2.6 Abl Kinases Regulate Tie2 Signaling

To evaluate the physiological consequences of Tie2 downregulation in endothelial cells lacking Abl kinases, we examined the activation of downstream cellular signaling pathways. As expected, treatment of control HUVECs with angiopoietin-1 led to tyrosine phosphorylation of the Tie2 receptor, along with activation of Akt and Erk pathways (**Figure 3.18 A,B**); activation of the PI3K/Akt pathway is required for Angpt1-mediated survival [173]. Notably, the Abl kinases were also activated following Angpt1 treatment, as evidenced by increased phospho-CrkL (Y207) levels (**Figure 3.18A**, *lanes 1 and 7*). Abl kinase activation similarly was observed following Angpt1 stimulation of both immortalized human microvascular endothelial cells (HMVECs; **Figure 3.18C**, *lanes 1 and 4*) and PyMT-immortalized mouse embryo endothelial cells (**Figure 3.19**, *lanes 1-6*). These findings suggest a potential role for the Abl/Arg kinases in mediating downstream Tie2 signaling. In this regard, activation of Akt by Angpt1 was dramatically reduced in *Abl*/*Arg*-knockdown cells (**Figure 3.18A**, *lanes 7-9*). Erk activation also was decreased to a lesser extent by Abl/Arg depletion. Similarly, *Abl*/*Arg* knockdown

diminished Angpt1-mediated Akt activation in HMVECs, while Erk activation was unchanged (**Figure 3.18C**, *lanes 4-6*).

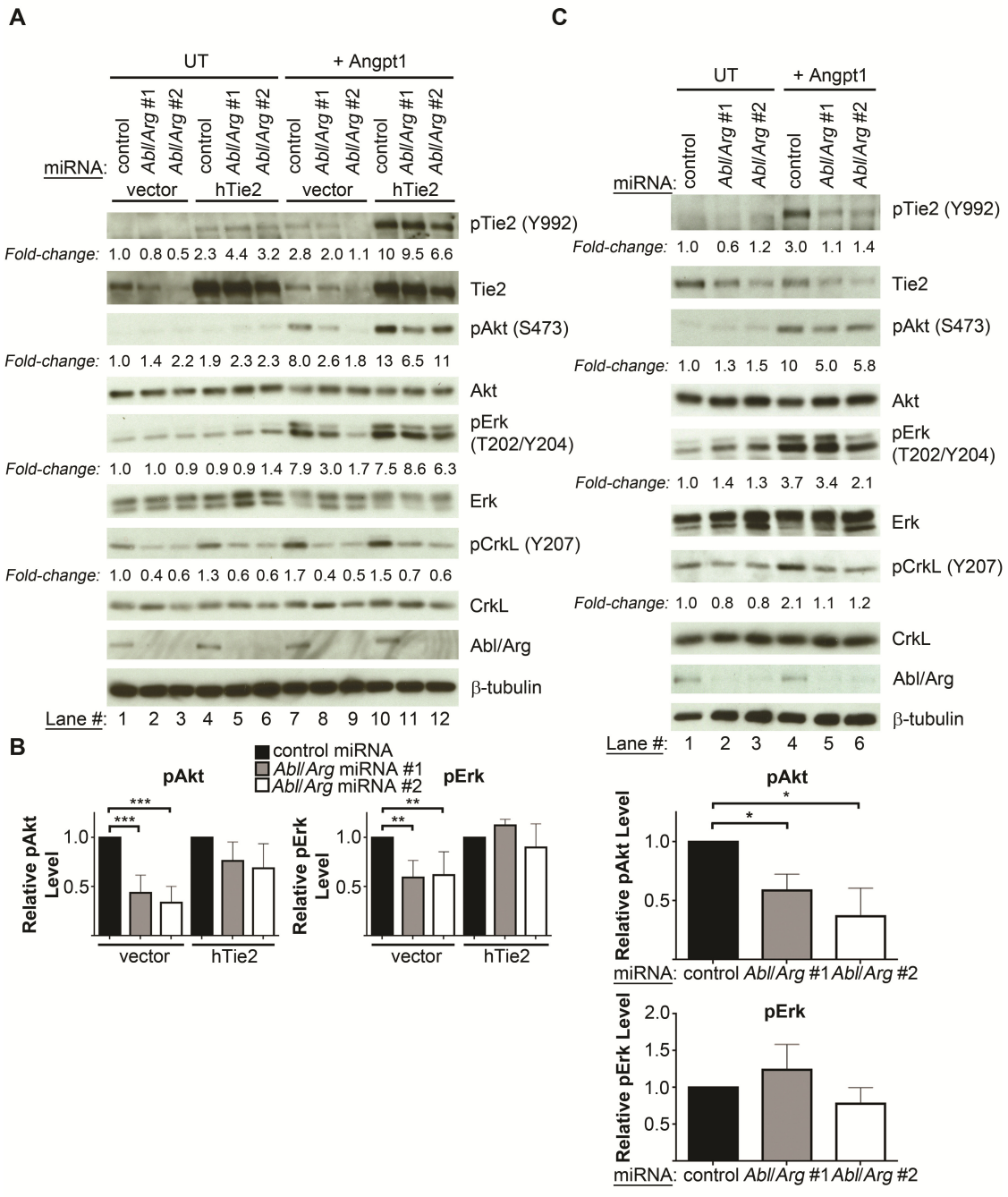

Figure 3.18 Abl Kinases Modulate Angpt1 Signaling

(**A** and **B**) Assessment of Angpt1-mediated activation of intracellular signaling pathways in HUVECs infected with control or *Abl*/*Arg* miRNA lentiviruses, with or without exogenous hTie2 expression, quantified in (**B**). Cells were serum-starved for 6 hours, then left unstimulated (UT) or treated with Angpt1 (200ng/mL, 15 minutes). (**B**) pAkt and pErk levels (normalized to total Akt and Erk protein) are shown as means +/- SD, relative to levels in Angpt1-stimulated control miRNA-expressing cells (n=4; **P<0.01; ***P<0.001). (**C**) Assessment of angiopoietin-1 (Angpt1)-mediated activation of intracellular signaling pathways in immortalized human microvascular endothelial cells (HMVECs) infected with control or *Abl*/*Arg* miRNA lentiviruses, quantified in bottom panels. Cells were serum-starved for 6 hours, then left unstimulated (UT) or treated with Angpt1 (200ng/mL, 15 minutes). pAkt and pErk levels (normalized to total Akt and Erk protein) are shown as means +/- SD, relative to levels in Angpt1-stimulated control miRNA-expressing cells (n=3; *P<0.05).

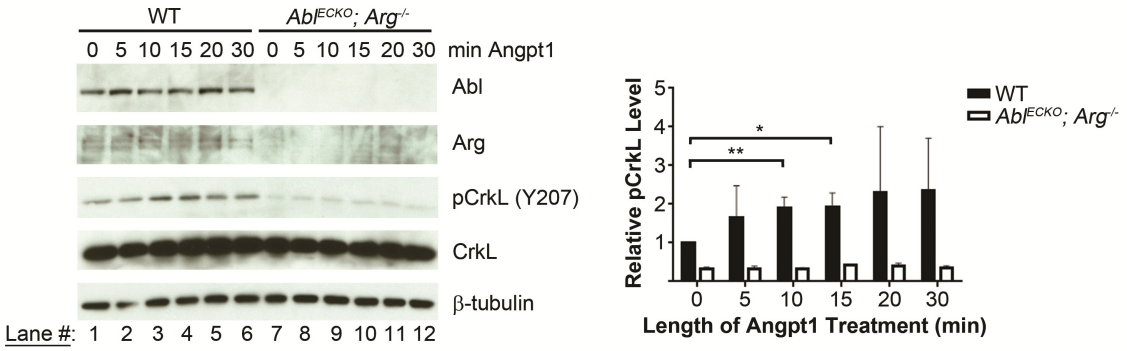

Figure 3.19 Abl Kinases Are Activated Following Angpt1 Stimulation

Assessment of timecourse of Angpt1-mediated activation of Abl kinases, as determined by phospho-CrkL (Y207) levels, in PyMT-immortalized endothelial cell lines from *Abl*$^{flox/flox}$; *Arg*$^{+/+}$; *Tie2-Cre*$^{-/-}$ (wild-type, WT) and *Abl*^{ECKO}; *Arg*$^{-/-}$ embryos. Fold-changes in pCrkL levels (normalized to total CrkL protein) are quantified in the right panel (means +/- SD, n=3). Angpt1 treatment increased pCrkL levels in WT, but not *Abl*^{ECKO}; *Arg*$^{-/-}$, endothelial cells. (*P<0.05; **P<0.01).

Importantly, while Angpt1 inhibited apoptosis following serum-starvation in control HUVECs, the pro-survival effects of Angpt1 were decreased in *Abl*/*Arg* knockdown cells (**Figure 3.20A**). Thus, downregulation of Tie2 receptor levels following

Abl/*Arg*-knockdown decreased both Angpt1-mediated signaling and downstream anti-apoptotic responses. Single *Abl* and *Arg* knockdowns demonstrated that loss of either kinase was sufficient to impair both Angpt1-mediated signaling (**Figure 3.20B**) and survival (**Figure 3.20C**). Interestingly, expression of exogenous Tie2 in *Abl*/*Arg*-knockdown cells largely restored Angpt1-mediated signaling (**Figure 3.18A**, *lanes 10-12*) and partially rescued the anti-apoptotic effects of Angpt1 (**Figure 3.20A**). These findings suggest that the increased apoptosis observed upon loss of Abl kinase expression may be due in part to downregulation of Tie2 signaling.

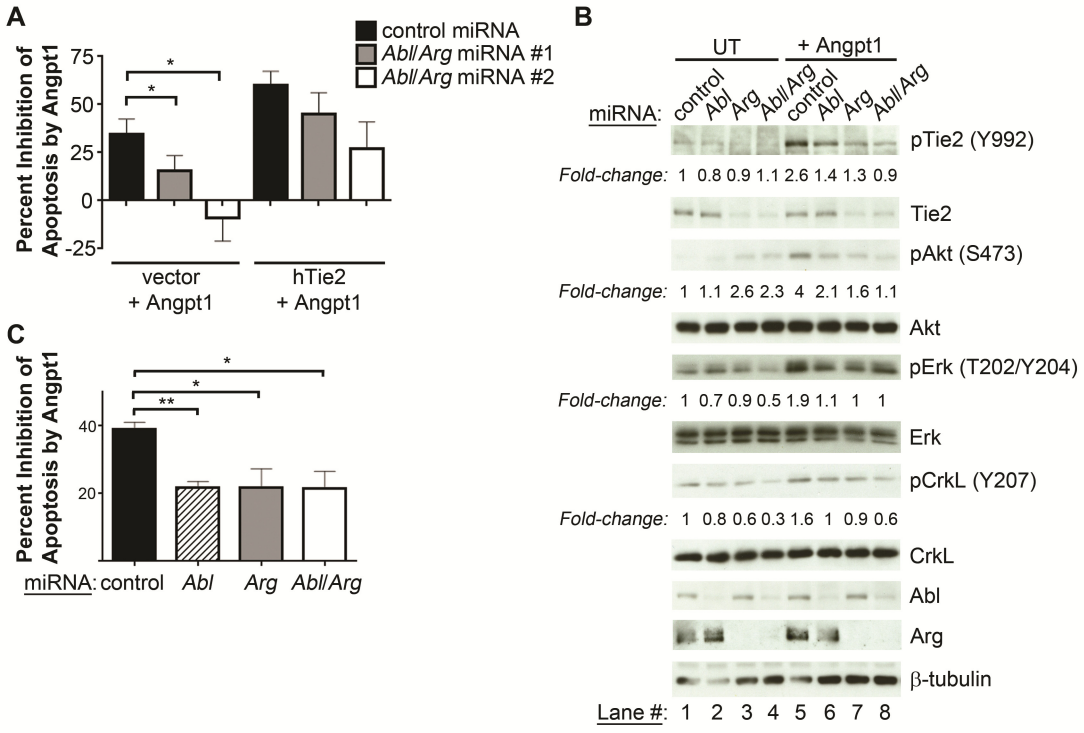

Figure 3.20 Abl and Arg Kinases Are Required for Angpt1-Mediated Survival

(**A**) Analysis of levels of apoptosis in HUVECs infected with Tie2 retrovirus and *Abl*/*Arg* miRNAs following 24 hours serum-starvation in the presence of Angpt1 (200ng/mL).

Values are expressed as percent inhibition of apoptosis by Angpt1 relative to serum-starved (non-supplemented) control cells. Data are presented as means +/- SEM (n=3; *P<0.05). (**B**) Assessment of Angpt1-mediated activation of intracellular signaling pathways in HUVECs expressing control miRNA, individual *Abl* or *Arg* miRNAs, or dual *Abl*/*Arg* miRNA. Fold-changes in pathway activation (normalized to total protein levels) are shown, expressed relative to unstimulated (UT) control miRNA-expressing cells. (**C**) Analysis of levels of apoptosis in HUVECs after 24 hours serum-starvation in the presence of Angpt1 (200ng/mL) following either *Abl* or *Arg* individual knockdown or dual knockdown of both *Abl*/*Arg* kinases. Values are expressed as percent inhibition of apoptosis by Angpt1 relative to serum-starved (non-supplemented) cells. Data are presented as means +/- SEM (n=3; *P<0.05; **P<0.01).

3.3 Discussion

Our findings have uncovered a crucial role for the endothelial Abl kinases in the vasculature, as loss of endothelial *Abl*/*Arg* kinase expression resulted in embryonic and perinatal lethality. Loss of endothelial Abl kinases adversely impacted vascular function, leading to localized loss of vascular density and resultant cell death in affected tissues (necrosis/apoptosis). Interestingly, even partial loss of endothelial Abl kinase expression produced focal loss of cardiac vasculature and myocardial injury. The localized nature of the observed vascular loss and tissue damage, along with the normal overall vascular density, branching, and patterning observed in Abl^{ECKO}; $Arg^{-/-}$ mice, suggest that loss of endothelial Abl/Arg kinases likely adversely affects vascular maintenance and stability, rather than vessel formation. Given that *Tie2-Cre*-mediated recombination occurs in most endothelial cells by E9.5 [367], it is possible that subtle structural defects during vessel formation could contribute to the phenotypes observed later in development in mutant embryos. However, our finding that loss of the Abl kinases sensitizes endothelial

cells to stress-induced apoptosis *in vitro* suggests that the sporadic and focal nature of the observed phenotypes may result from vascular damage due to localized endothelial cell apoptosis in response to cumulative vascular stresses in the absence of the Abl kinases.

Our demonstration of a critical requirement for the Abl kinases in the vasculature is particularly notable considering the cardiotoxicity previously observed in a subset of patients upon chronic Abl kinase inhibition using imatinib [350,351,352]. Additional case reports detail instances of interstitial lung disease of unknown origin in some imatinib-treated cancer patients [353]. Although the incidence of these events appears to be low, and imatinib is generally well tolerated [368], our findings demonstrate a crucial role for the Abl kinases in normal vascular development and function, which may have implications for the clinical use of Abl kinase inhibitors such as imatinib and nilotinib.

Using both pharmacological inhibition and knockdown studies, we found a requirement for Abl kinases in promoting endothelial cell survival, both under stress conditions and in the presence of specific angiogenic factors. Consistent with a previous report, Abl kinase activity was required for maximal bFGF-mediated endothelial survival [337]. However, we also observed a requirement for Abl family kinases during VEGF-mediated endothelial cell survival. Imatinib has shown anti-angiogenic activity in several *in vivo* models [334,335,336]. While these anti-angiogenic actions have been

attributed largely to the inhibition of PDGFR activity, which is necessary for endothelial cell interactions with supporting mural cells, our findings that the Abl kinases modulate endothelial survival in response to pro-angiogenic growth factors (angiopoietin-1, VEGF, and bFGF) suggest that the endothelial Abl kinases may be additional anti-angiogenic targets of imatinib.

Previous studies have established both anti- and pro-apoptotic roles for the Abl kinases in various cell types. Global *Abl*/*Arg* knockout embryos exhibited increased apoptosis in all tissues [329]. Enhanced apoptosis susceptibility similarly was observed in *Abl*-null thymocytes [332], as well as following serum-deprivation in *Abl*-deficient B cell lines [369]. However, while Abl kinases are activated upon growth factor stimulation in various cell types [279,337], Abl also is activated following exposure to ionizing radiation or chemotherapeutic agents and is required for apoptosis induced by these stresses [370], suggesting a pro-apoptotic role for the Abl kinases. Our findings, however, suggest a physiological anti-apoptotic role for the Abl kinases in the endothelium, which may be important clinically, as endothelial cell apoptosis has been reported in a number of vascular pathologies [371].

Unexpectedly, the current study also reveals bi-directional links between the Abl kinases and pro-survival angiopoietin/Tie2 signaling in the endothelium. Loss of endothelial *Abl*/*Arg* kinase expression decreased Tie2 receptor levels and led to a shift in angiopoietin levels, with enhanced *Angpt2* levels and decreased *Angpt1* levels.

Consequently, loss of Abl kinases decreased Angpt1/Tie2 signaling and diminished the pro-survival effects of Angpt1. Our finding that Abl kinases are activated following Angpt1 stimulation supports a dual role for Abl kinases in the regulation of angiopoietin/Tie2 signaling, through the control of receptor/ligand expression, as well as the modulation of downstream pro-survival signaling pathways (**Figure 3.21**). Loss of Tie2 impairs endothelial cell survival *in vivo* [160]. Angpt1/Tie2 signaling also supports vascular stability and inhibits inflammatory endothelial barrier dysfunction and adhesion molecule expression [372]. It will be of interest to determine whether, in addition to the observed effects on Angpt1-mediated survival, the Abl kinases also may modulate other endothelial cell responses to Angpt1, including anti-permeability and anti-inflammatory effects.

Taken together, our findings support an important role for the Abl kinases in Angpt1/Tie2-mediated vascular homeostasis. Alterations in the angiopoietin/Tie2 pathway, including a shift in angiopoietin balance with Angpt2 levels exceeding Angpt1 levels, have been implicated in diverse vascular pathologies [373]. Thus, future studies are required to evaluate the role of the Abl kinases in the modulation of Angpt/Tie2 signaling during the progression of these disorders, in order to fully understand the role of these kinases in both vascular development and adult vascular maintenance.

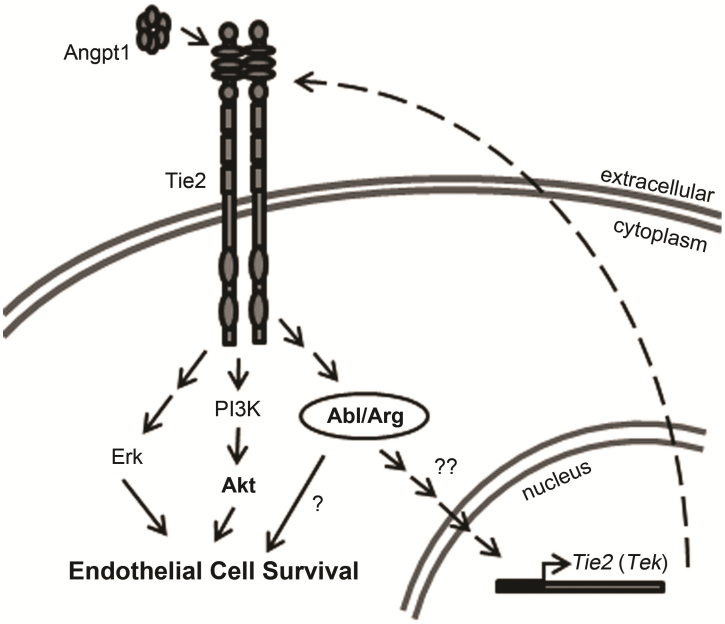

Figure 3.21 Model for the Dual Role of the Abl Kinases in Angiopoietin/Tie2 Signaling

The Abl kinases positively regulate *Tie2* (*Tek*) mRNA expression and are required for maximal Angpt1-mediated pro-survival signaling primarily through the PI3K/Akt and, to a lesser extent, Erk signaling pathways. The Abl kinases are also activated downstream of the Tie2 receptor.

4. Abl Family Kinases Regulate Endothelial Barrier Function *In Vitro* and in Mice

4.1 Introduction

The endothelium forms a critical semi-permeable barrier between tissues and the bloodstream, regulating the transport of solutes and immune cells into and out of the circulation. The maintenance of this barrier is a dynamic and tightly-controlled process. Loosening of the endothelial barrier is induced by a variety of soluble factors, including cytokines and other inflammatory mediators, as well as VEGF, and is an important aspect of both normal angiogenic remodeling and inflammatory responses [5,30]. However, abnormally elevated vascular permeability is a key feature of a variety of pathological conditions, including cancer, sepsis, and ischemia-reperfusion injury [31,32]. This uncontrolled vascular leakage can lead to edema, increased interstitial fluid pressure, and tissue damage [32].

Vascular permeability can occur through both transcellular and paracellular mechanisms. In the transcellular pathway, solutes or cells pass through individual endothelial cells via vesicular transport mechanisms [374]. In contrast, paracellular permeability requires the dynamic opening and closure of inter-endothelial cell-cell adherens and tight junctions, enabling the passage of plasma molecules or cells between neighboring endothelial cells [41]. The transmembrane protein VE-cadherin is the major structural component of endothelial adherens junctions and is a critical regulator of vascular integrity and endothelial barrier function [44]. Dimerization and clustering of

VE-cadherin at sites of endothelial cell-cell contact leads to homotypic, Ca^{2+}-dependent interaction of the extracellular domains of VE-cadherin proteins on neighboring cells, which are then linked indirectly to the actin cytoskeleton through the binding of the VE-cadherin intracellular domain to β-catenin and α-catenin proteins [45].

The endothelial adherens junction complex is targeted by a variety of vascular permeability-inducing factors, including VEGF, thrombin, and histamine. Stimulation of endothelial cells with these barrier-disruptive factors leads to dissolution of cell-cell junctions through mechanisms including VE-cadherin internalization, destabilization of adherens junction protein complexes, or reduced association of VE-cadherin complexes with the actin cytoskeleton [106,122,375,376,377]. In addition to direct effects on cell-cell junctions, endothelial barrier-disrupting factors increase acto-myosin contractility and centripetal tension, which weakens intercellular adhesion and can lead to cell retraction and formation of intercellular gaps [378,379]. Tyrosine phosphorylation has been implicated in the destabilization of the endothelial barrier by a variety of permeability-inducing factors. Increased tyrosine phosphorylation of adherens junction proteins including VE-cadherin and β-catenin has been observed following VEGF, histamine, and thrombin stimulation [105,375,380]; this phosphorylation has been linked to destabilization of cell-cell adhesion. Additionally, treatment with tyrosine kinase inhibitors decreased endothelial permeability induced by each of these agonists

[92,375,378,381], demonstrating an important role for tyrosine kinases in the induction of endothelial barrier dysfunction.

Our previous work has demonstrated a role for the Abl family kinases (Abl and Arg) both in formation and maintenance of epithelial adherens junctions [294], suggesting a potential role for these kinases in the regulation of barrier function. Interestingly, treatment with the Abl kinase pharmacological inhibitor imatinib (Gleevec) decreased interstitial fluid pressure in lung and colon cancer models, resulting in improved tumor oxygenation and drug delivery [340,341,342]. Imatinib treatment also reduced permeability following administration of thrombolytic tissue plasminogen activator in a murine model of ischemic stroke [343], suggesting a beneficial effect of imatinib on vascular barrier function. Pre-treatment with imatinib (or the more potent Abl kinase inhibitor nilotinib) similarly protected against pulmonary edema following lipopolysaccharide-induced acute lung injury in mice [344]. These protective effects have been attributed to the inhibition of the PDGF receptor (PDGFR), which is also targeted by imatinib [265]. However, recent studies have implicated the Abl kinases in the regulation of endothelial barrier function [345,346]. Expression of the Abl kinase is required for the endothelial barrier-promoting effects of sphingosine-1-phosphate *in vitro* [346]. Imatinib treatment protected against vascular leakage and edema in a murine sepsis model, which was attributed to the inhibition of the endothelial Arg kinase [345]. However, the *in vivo* protective effects of imatinib may result from inhibition of multiple

tyrosine kinases and targeting of cell types other than endothelial cells, including immune cells.

In the current study, we demonstrate a requirement for activation of the Abl kinases in endothelial permeability induced by VEGF and the inflammatory mediators thrombin and histamine. Use of Abl/Arg-specific pharmacological inhibitors or *Abl* knockdown impaired induction of endothelial permeability in response to these agonists *in vitro*. VEGF-induced permeability similarly was decreased following Abl kinase inhibition *in vivo*. Importantly, impaired VEGF-induced permeability was also observed in conditional knockout mice lacking endothelial *Abl* expression. Mechanistically, we demonstrate that Abl kinase inhibition both increased activation of the endothelial barrier-supporting GTPases Rac1 and Rap1 and decreased the activation of pathways regulating induction of acto-myosin contractility in response to permeability-inducing factors. Taken together, these findings demonstrate an important role for the Abl kinases in mediating endothelial barrier dysfunction induced by a variety of agonists and support the potential use of Abl kinase inhibitors in the treatment of disorders characterized by pathological vascular permeability.

4.2 Results

4.2.1 Abl Kinases Are Activated Following Treatment with Endothelial Permeability-Inducing Factors

Endothelial barrier dysfunction can be induced in response to a variety of soluble mediators [30]. To assess a potential role for the Abl kinases in the regulation of

endothelial barrier function, we initially evaluated Abl kinase activity following treatment of human microvascular endothelial cells (HMVECs) with the permeability-inducing factors VEGF, thrombin, and histamine. In agreement with previous findings in human umbilical vein endothelial cells (HUVECs) [338,345,382], stimulation of HMVECs with VEGF resulted in Abl kinase activation, as assessed by the phosphorylation of CrkL at tyrosine (Y) 207, an Abl-specific phosphorylation site [366] (**Figure 4.1A)**, which was prevented by pre-treatment with the ATP-competitive Abl kinase inhibitor imatinib. Interestingly, pre-treatment with the Src kinase inhibitor su6656 partially blocked Abl kinase activation in response to VEGF stimulation (**Figure 4.1B**), suggesting that the Abl kinases may act downstream of Src family kinases in VEGF-mediated signaling. Notably, Abl kinases were markedly activated by treatment of HMVECs with thrombin (**Figure 4.1C**) or histamine (**Figure 4.1D**). Thus, these findings demonstrate that the Abl kinases are activated in response to several distinct endothelial permeability-inducing mediators, suggesting a potential function for these kinases in mediating downstream permeability responses.

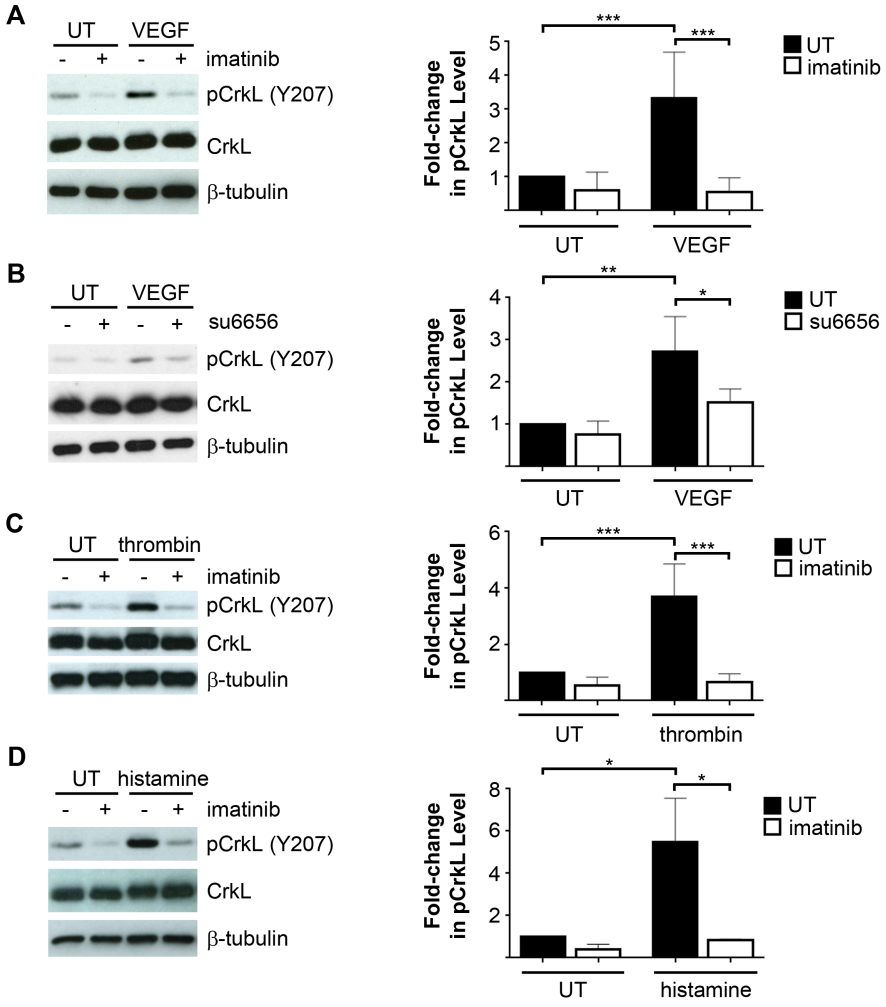

Figure 4.1 Abl kinases Are Activated Following Treatment with Endothelial Permeability-Inducing Factors

(**A**) Assessment of Abl kinase activation, as determined by phospho-CrkL tyrosine (Y) 207 levels, following stimulation of serum-starved HMVECs with 100ng/mL VEGF for 5 minutes, with or without imatinib pre-treatment (10μM). pCrkL (Y207) levels (normalized to total CrkL) are quantified in the right panel, relative to levels in untreated (UT) cells. Data are presented as means +/- SD (n=7). (**B**) Evaluation of pCrkL (Y207) levels in HMVECs treated with VEGF, with or without su6656 pre-treatment (1μM). pCrkL levels (normalized to total CrkL) are quantified in the right panel, relative to levels in untreated (UT) cells. Data are presented as means +/- SD (n=2). *P<0.05; **P<0.01; ***P<0.001. (**C**) Evaluation of Abl kinase activation (pCrkL Y207) following treatment of HMVECs with thrombin (1U/mL, 5 minutes), with or without imatinib pre-

treatment. pCrkL levels (normalized to total CrkL) are quantified in the right panel, relative to levels in untreated (UT) cells. Data are presented as means +/- SD (n=5). (**D**) Assessment of Abl kinase activation (pCrkL Y207) following stimulation of HMVECs with histamine (100μM, 5 minutes), with or without imatinib pre-treatment. pCrkL levels (normalized to total CrkL) are quantified in the right panel, relative to levels in untreated (UT) cells. Data are presented as means +/- SD (n=3).

4.2.2 Loss of Abl Kinase Function Decreased Endothelial Permeability *In Vitro*

We examined whether the Abl kinases may play a role in the induction of endothelial permeability *in vitro*, by assessing the passage of fluorescein-labeled dextran through HMVEC monolayers following pharmacological inhibition of the Abl kinases. Consistent with previous reports [99,345], Abl kinase inhibition with imatinib greatly decreased endothelial barrier dysfunction induced by VEGF (**Figure 4.2A**,**B**). Imatinib similarly inhibited permeability induced by thrombin and histamine (**Figure 4.2B**). As imatinib also inhibits kinases other than Abl and Arg, including the receptors c-Fms, PDGFR, Kit, and the discoidin domain receptors [265,267,268], we examined the effects of the allosteric Abl kinase inhibitor GNF-2 on endothelial permeability. GNF-2, which binds to the myristate-binding pocket in the kinase domain of Abl and Arg, displays greater target specificity than imatinib and is not known to inhibit any additional kinases [274,275]. Importantly, the Abl/Arg-specific inhibitor GNF-2 also decreased endothelial permeability induced by VEGF, thrombin, or histamine (**Figure 4.2B**), suggesting that the preservation of endothelial barrier function upon imatinib treatment likely results from inhibition of the Abl family kinases.

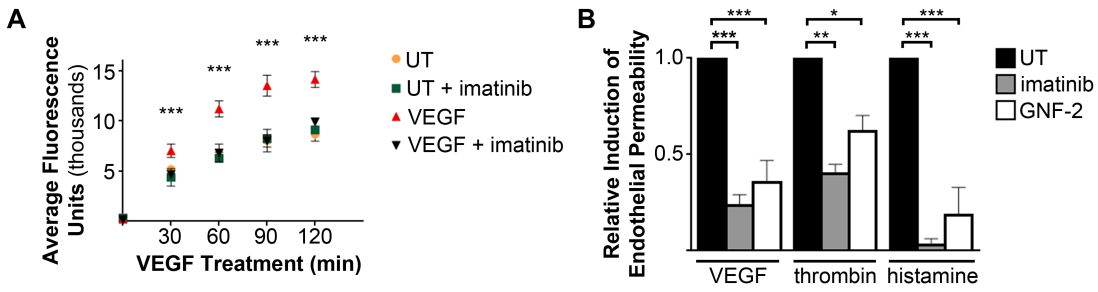

Figure 4.2 Abl Kinase Inhibition Decreased Endothelial Permeability *In Vitro*

(**A**) Evaluation of endothelial monolayer permeability, as assessed by passage of fluorescein-labeled dextran (molecular weight 40kDa) through HMVEC monolayers grown on Transwells, following treatment with VEGF (100ng/mL) with or without imatinib pre-treatment (10μM). Data shown are mean fluorescence of samples collected from bottom Transwell chambers at the indicated times post-VEGF treatment, +/- SD of three replicates per treatment. Data are representative of three independent experiments. (**B**) Quantification of inhibition of endothelial monolayer permeability to fluorescein-labeled dextran by imatinib (10μM) or GNF-2 (15μM). Endothelial barrier dysfunction was induced by treatment of HMVECs with VEGF (100ng/mL, 120 minutes), thrombin (1U/mL, 30 minutes) or histamine (100μM, 60 minutes). Values are expressed relative to permeability induction in vehicle-treated cells (UT). Data are presented as means +/- SEM (n=3). (*P<0.05; **P<0.01; ***P<0.001).

To directly evaluate whether Abl is implicated in the regulation of endothelial permeability, we depleted Abl expression in HMVECs. VEGF-induced endothelial permeability was inhibited by micro-RNA (miRNA)-mediated *Abl* knockdown (**Figure 4.3A,C,E**), and permeability induction was restored by re-expression of miRNA-resistant Abl (**Figure 4.3A**,**B**). A similar reduction of thrombin-induced endothelial barrier dysfunction was observed upon *Abl* knockdown (**Figure 4.3D**,**E**). Interestingly, Abl kinase inhibition using either imatinib or GNF-2 more potently inhibited endothelial permeability responses than did *Abl* knockdown alone (**Figure 4.2B** vs. **Figure 4.3E**),

suggesting that Arg may also contribute to VEGF- and thrombin-induced endothelial permeability.

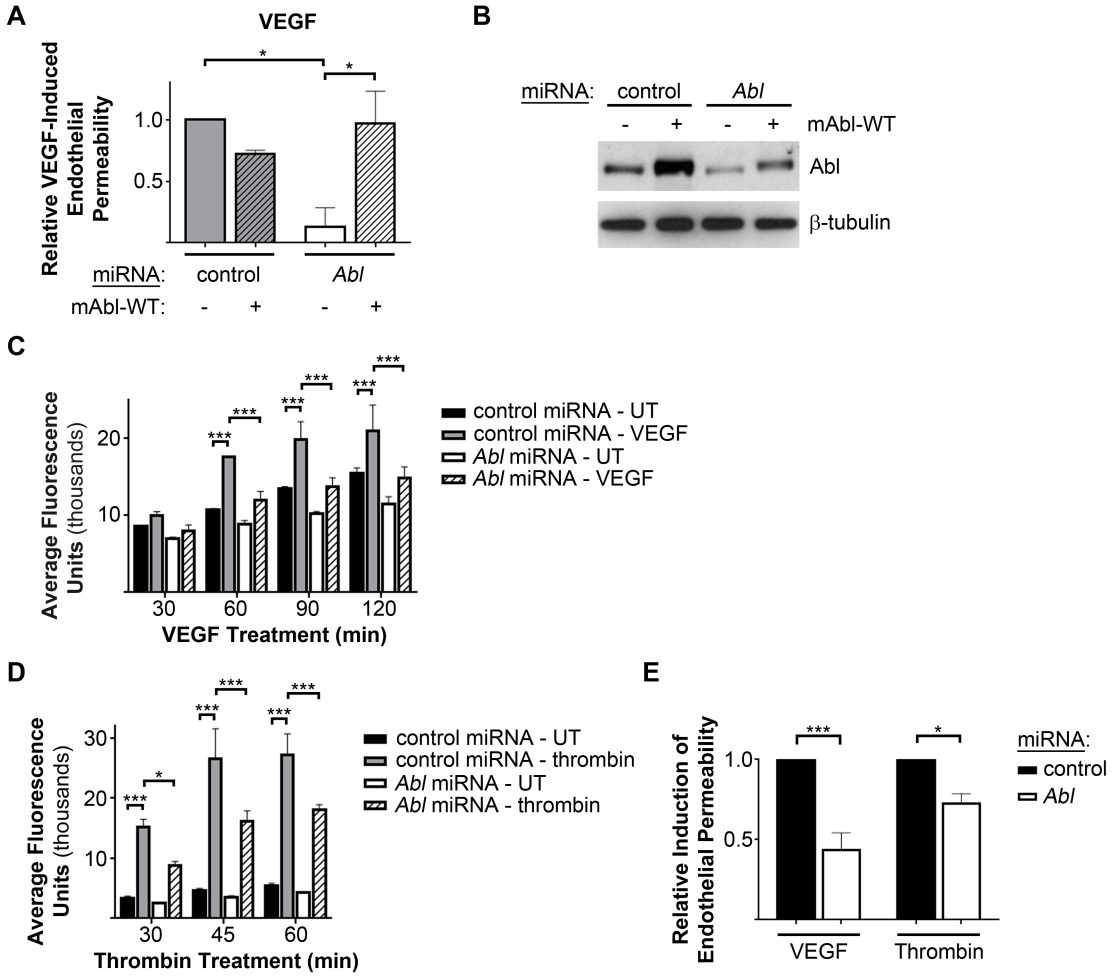

Figure 4.3 *Abl* Knockdown Impaired VEGF- and Thrombin-Induced Endothelial Permeability

(**A**) Quantification of VEGF-induced endothelial permeability of HMVECs expressing either control or *Abl* miRNAs, with or without re-expression of miRNA-resistant, wild-type murine Abl (mAbl-WT). Values are expressed relative to VEGF-induced permeability in control miRNA-expressing cells. Data are presented as means +/- SEM (n=3). (**B**) Assessment of Abl protein levels following miRNA expression in HMVECs, with or without re-expression of miRNA-resistant Abl. (**C** and **D**) Assessment of permeability of HMVEC monolayers expressing either control or *Abl* miRNAs to

fluorescein-labeled dextran (molecular weight 40kDa), following (**C**) VEGF (100ng/mL) or (**D**) thrombin (1U/mL) treatment. Data shown are mean fluorescence of samples collected from bottom Transwell chambers at the indicated times following VEGF or thrombin treatment, +/- SD of three replicates per treatment. Data are representative of 4-5 independent experiments. (**E**) Quantification of inhibition of VEGF- and thrombin-induced endothelial permeability following *Abl* knockdown. Values are expressed relative to permeability of HMVECs expressing control miRNA. Data are presented as means +/- SEM (VEGF, n=5; thrombin, n=4). (*P<0.05; **P<0.01; ***P<0.001).

However, dual knockdown of both Abl and Arg kinases led to a nearly twofold increase in baseline permeability of unstimulated HMVEC monolayers (**Figure 4.4A**,**B**), which precluded the analysis of VEGF- and thrombin-induced permeability. These data are consistent with our finding that depletion of both Abl and Arg proteins induces dissolution of adherens junctions in epithelial cells [294]. In all, these results demonstrate a requirement for Abl kinase activity, as well as Abl expression, in endothelial barrier dysfunction induced by several permeability-inducing factors.

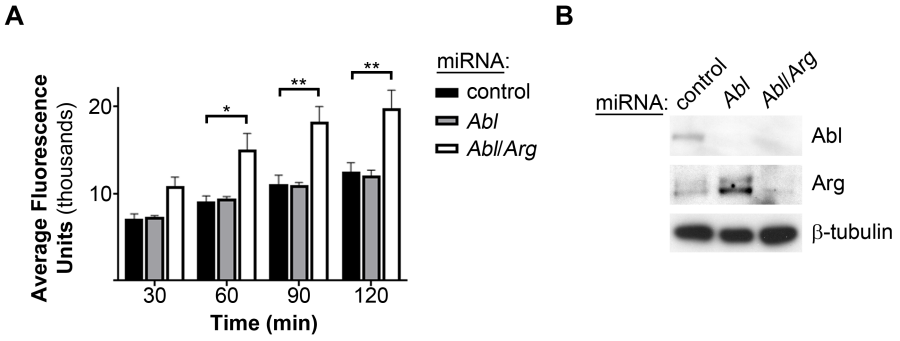

Figure 4.4 Increased Baseline Endothelial Permeability Following *Abl*/*Arg* Knockdown

(**A**) Evaluation of baseline permeability to fluorescein-labeled dextran of unstimulated HMVEC monolayers expressing control, *Abl*, or *Abl*/*Arg* miRNAs. Data are presented as

means +/- SEM (n=4). (**B**) Assessment of Abl and Arg protein levels in HMVECs following *Abl* or *Abl*/*Arg* knockdown. (*P<0.05; **P<0.01).

4.2.3 Abl Kinase Activity Is Required for VEGF-Induced Permeability *In Vivo*

To evaluate whether Abl kinases are involved in VEGF-induced vascular permeability *in vivo*, we employed both pharmacological inhibition and genetic inactivation of endothelial *Abl* kinase in mice. Consistent with our *in vitro* findings, inhibition of the Abl kinases with either imatinib or GNF-2 decreased VEGF-induced vascular leakage of albumin (by approximately 30% and 50%, respectively), as assessed by extravasation of intravenously-administered Evans blue dye following intradermal administration of VEGF (**Figure 4.5A**).

To directly assess the role of the Abl kinases in VEGF-induced permeability *in vivo*, we generated conditional knockout mice lacking Abl kinase expression in the endothelium, by crossing mice carrying a floxed *Abl* allele ($Abl^{flox/flox}$) on an $Arg^{-/-}$ background to *Tie2-Cre* mice [382]. As loss of both endothelial *Abl* and *Arg* expression ($Abl^{flox/flox}$; $Arg^{-/-}$; $Tie2\text{-}Cre^{+/-}$) resulted in late-stage embryonic and perinatal lethality [382], we instead examined permeability responses using endothelial *Abl* knockout mice on an $Arg^{+/-}$ background ($Abl^{flox/flox}$; $Arg^{+/-}$; $Tie2\text{-}Cre^{+/-}$, referred to as Abl^{ECKO}; $Arg^{+/-}$), which survive to adulthood. Notably, VEGF-induced vascular permeability was reduced in Abl^{ECKO}; $Arg^{+/-}$ mice (**Figure 4.5B**). While VEGF induced a two-fold increase in Evans blue dye extravasation in $Arg^{+/-}$ control mice ($Abl^{flox/flox}$; $Arg^{+/-}$; $Tie2\text{-}Cre^{-/-}$), no significant increase in

vascular leakage was observed following VEGF treatment in *Abl*^{ECKO}*; Arg*$^{+/-}$ mice. A previous report suggested that Arg, rather than Abl, mediates the *in vitro* endothelial barrier-enhancing effects of imatinib [345]. However, our genetic results show that Abl is a critical player in the regulation of endothelial barrier function *in vivo*. Taken together, these findings demonstrate a requirement for the Abl family kinases in VEGF-induced vascular permeability *in vivo*.

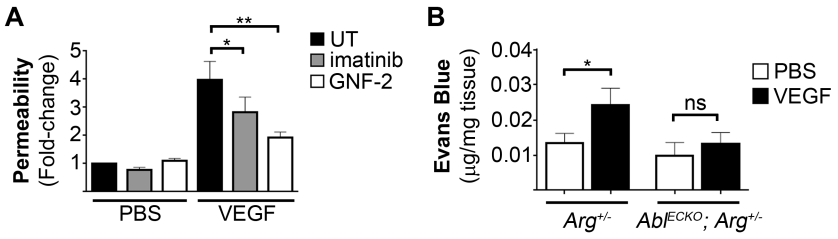

Figure 4.5 Abl Kinases Are Required for VEGF-Induced Vascular Permeability *In Vivo*

(**A**) Evaluation of vascular leakage of Evans blue dye in mice following intradermal injection of VEGF (100ng, 15 minutes) with or without concomitant treatment with imatinib or GNF-2 (15µM). Results are expressed as fold-change in permeability relative to phosphate-buffered saline (PBS) control (UT). Data are presented as means +/- SD (n=12). (**B**) Quantification of VEGF-induced dermal vascular leakage of Evans blue dye in *Abl*^{ECKO}*; Arg*$^{+/-}$ (*Abl*$^{flox/flox}$*; Arg*$^{+/-}$*; Tie2-Cre*$^{+/-}$) and age/sex-matched *Arg*$^{+/-}$ control mice (*Abl*$^{flox/flox}$*; Arg*$^{+/-}$*; Tie2-Cre*$^{-/-}$). Dye extravasation was normalized to tissue weight. Values are presented as means +/- SD (*Arg*$^{+/-}$ controls, n=8; *Abl*^{ECKO}*; Arg*$^{+/-}$, n=6). (*P<0.05; **P<0.01).

4.2.4 Abl Kinase Activity Is Required for VEGF- and Thrombin-Induced Remodeling of Endothelial Adherens Junctions

Induction of endothelial barrier dysfunction has previously been linked to disruption of endothelial cell-cell adhesion, through the phosphorylation and disruption

of endothelial adherens junction complexes, as well as VE-cadherin mislocalization and internalization [106,122,375,376,377]. As the Abl kinases are required for adherens junction formation and mediate signaling downstream of cadherin engagement in epithelial cells [294], we examined whether the Abl kinases might regulate VE-cadherin dynamics in endothelial cells following stimulation with permeability-inducing factors. While a continuous pattern of VE-cadherin staining was observed at endothelial cell-cell junctions in unstimulated cells, both VEGF and thrombin treatment disrupted VE-cadherin localization, leading to destabilization of endothelial adherens junctions ("zig-zag" VE-cadherin staining pattern, arrowheads) and formation of inter-endothelial cell gaps (arrows) (**Figure 4.6A**-**C**). Consistent with their anti-permeability effects, pre-treatment with the Abl kinase inhibitors imatinib (**Figure 4.6A**,**B**) or GNF-2 (**Figure 4.6C**) reduced the VEGF- and thrombin-induced disruption of VE-cadherin localization.

However, imatinib treatment did not alter VE-cadherin cell-surface levels (**Figure 4.7A**) or association with catenin proteins (**Figure 4.7B**-**E**). VEGF-induced endothelial permeability previously has been linked to tyrosine phosphorylation of adherens junction proteins including VE-cadherin and β-catenin, which is thought to destabilize cellular adherens junctions [41,105,376]. However, we did not observe changes in VE-cadherin or β-catenin tyrosine phosphorylation in response to VEGF stimulation, either in the presence or absence of imatinib treatment (data not shown). Taken together, these findings suggest that Abl kinase inhibition prevents VEGF- and thrombin-induced

disruption of endothelial adherens junctions independently of direct effects on VE-cadherin and other adherens junction components.

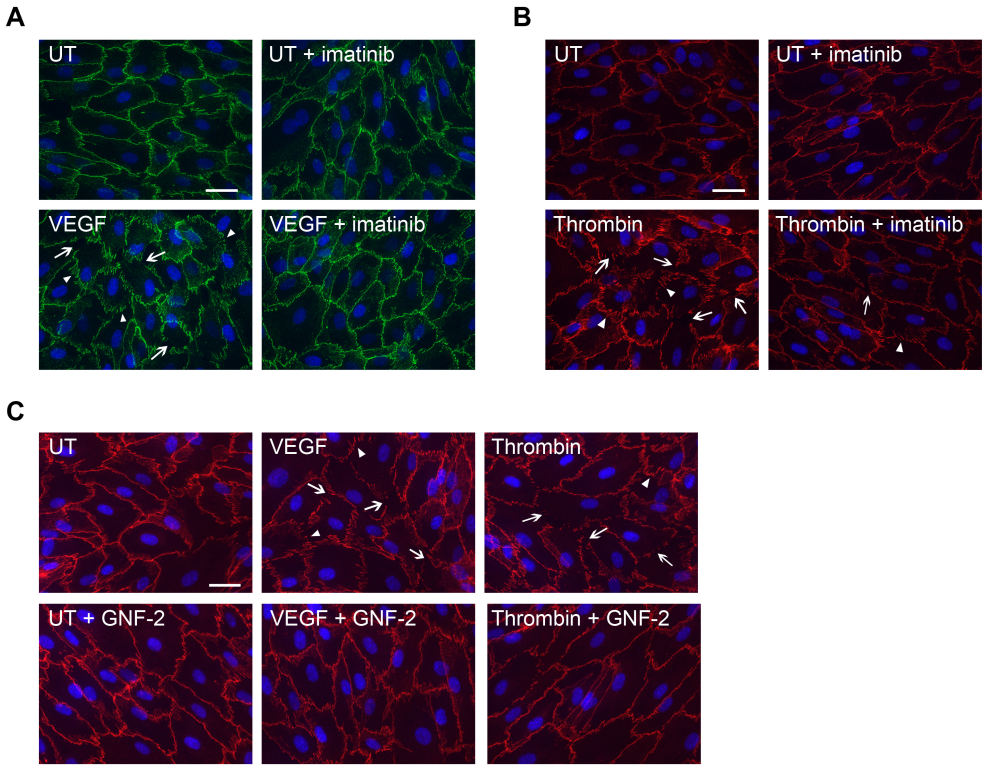

Figure 4.6 Abl Kinase Activity Is Required for VEGF- and Thrombin-Induced Remodeling of Endothelial Adherens Junctions

(**A**) Staining of HMVEC monolayers for the adherens junction marker VE-cadherin (green) following treatment with VEGF (100ng/mL, 30 minutes), with or without imatinib pre-treatment (10μM). (**B**) VE-cadherin staining (red) of HMVEC monolayers treated with thrombin (1U/mL, 5 minutes), +/- imatinib. (**C**) VE-cadherin staining (red) of VEGF or thrombin-treated HMVECs, with or without GNF-2 pre-treatment (15μM). VEGF and thrombin treatment induced formation of inter-endothelial cell gaps (arrows) and destabilization of endothelial cell-cell junctions ("zig-zag" VE-cadherin staining pattern, arrowheads), which were reduced by pre-treatment with Abl kinase inhibitors.

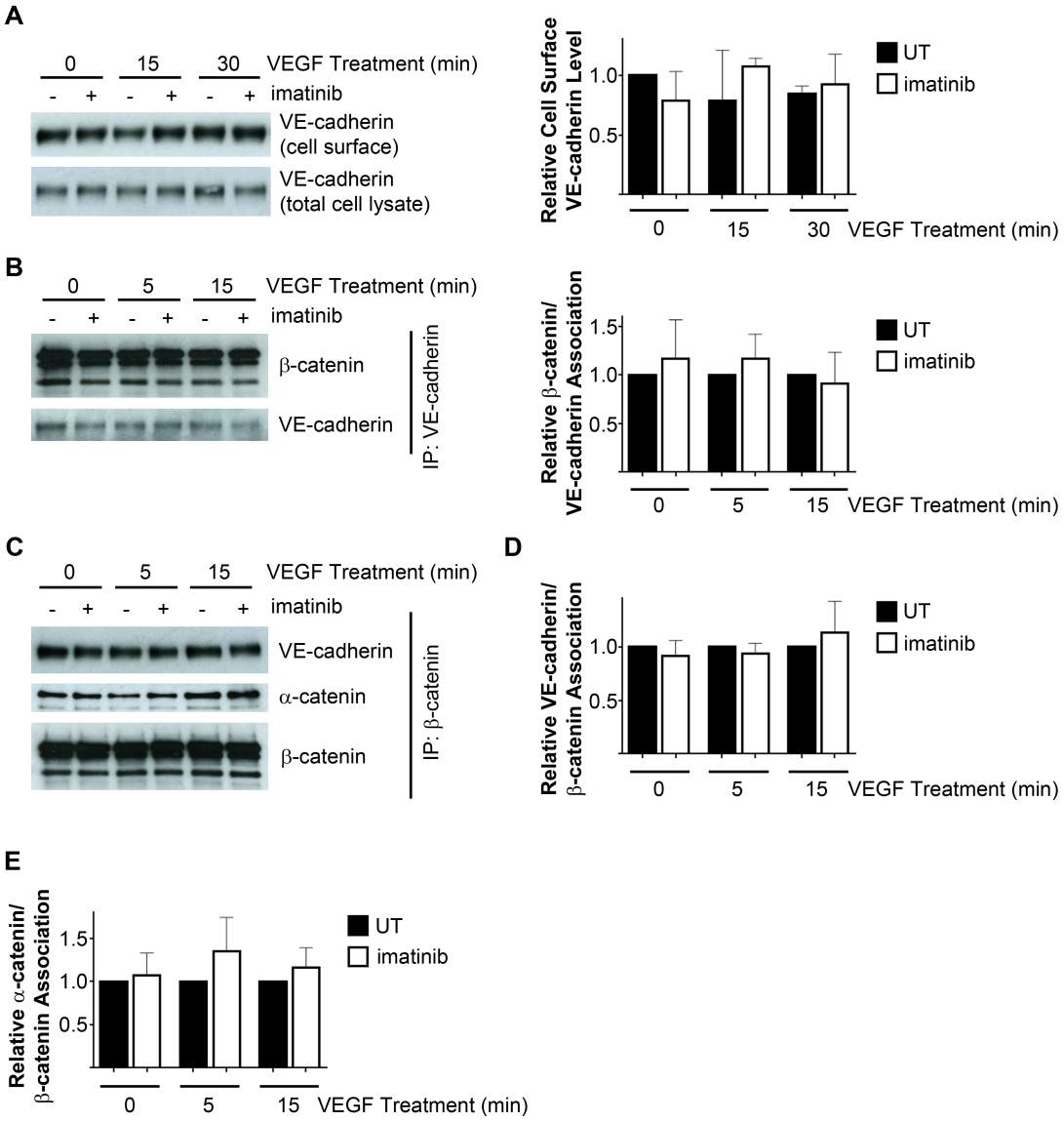

Figure 4.7 Abl Kinase Inhibition Did Not Alter VE-cadherin Cell Surface Levels or Adherens Junction Complex Association

(**A**) Evaluation of total and cell surface VE-cadherin protein levels in HMVECs treated with VEGF (100ng/mL) with or without imatinib pre-treatment (10μM), as assessed by biotinylation of cell surface proteins. Cell surface VE-cadherin levels are quantified in the right panel, relative to levels in untreated cells (UT). Data are presented as means +/- SD (n=3). (**B**) Assessment of VE-cadherin association with β-catenin in HMVECs treated with VEGF +/- imatinib, following VE-cadherin immunoprecipitation. Data are quantified in the right panel as means +/- SD (n=5), relative to co-immunoprecipitated β-catenin levels in vehicle-treated cells (UT) at each time point. (**C**-**E**) Assessment of β-

catenin association with VE-cadherin and α-catenin in HMVECs treated with VEGF +/- imatinib, following β-catenin immunoprecipitation. (**D** and **E**) Quantification of levels of co-immunoprecipitated (**D**) VE-cadherin and (**E**) α-catenin, relative to levels in vehicle-treated cells (UT) at each time point. Data are presented as means +/- SD (VE-cadherin, n=5; α-catenin, n=2).

4.2.5 Activation of Rac1 and Rap1 GTPases Following Abl Kinase Inhibition

Endothelial permeability and adherens junction stability are modulated by the activity of a variety of small GTPase proteins, which regulate cytoskeletal remodeling and act either to stabilize or disrupt barrier function [383]. Of these, the Rho family GTPase Rac1 and Ras family GTPase Rap1 have been identified as important mediators in the maintenance of endothelial barrier function. Rac1 activation opposes the induction of endothelial permeability, in part by remodeling of cortical actin and stabilizing adherens junctions [190,191,192]. Consistent with previously-reported findings [345], Abl kinase inhibition with imatinib increased the levels of active, GTP-bound Rac1 both in unstimulated and VEGF-treated HMVECs (**Figure 4.8A**,**B**). To examine the contribution of this increased Rac1 activation to the anti-permeability effects of imatinib, we examined endothelial monolayer permeability in HMVECs expressing *Rac1* shRNA (**Figure 4.8D**). As expected, and in line with the barrier-stabilizing effects of Rac1 activation, *Rac1* knockdown increased baseline permeability in unstimulated cells (**Figure 4.8C**). However, imatinib inhibited VEGF-induced permeability in HMVECs

after *Rac1* knockdown (**Figure 4.8C**), suggesting that Rac1-independent pathways mediate the endothelial barrier-stabilizing effects of Abl kinase inhibition.

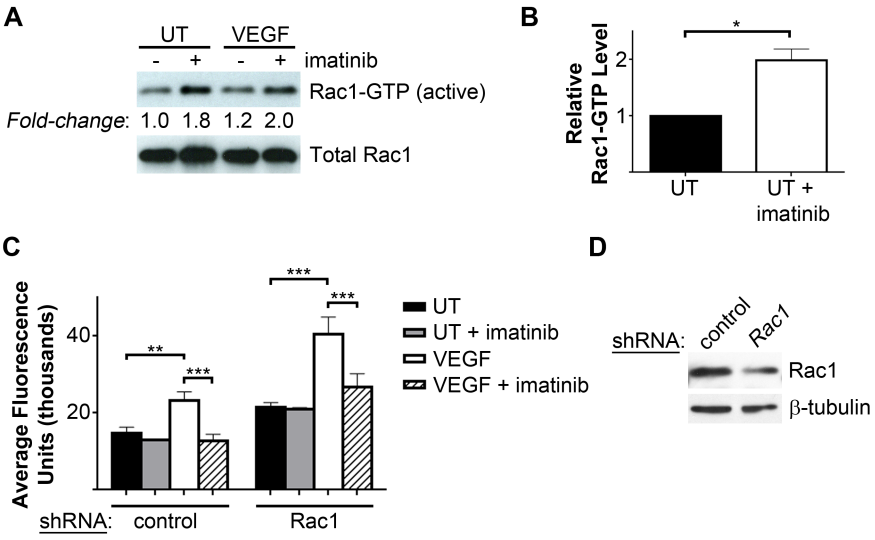

Figure 4.8 Increased Rac1 GTPase Activity Following Abl Kinase Inhibition

(**A** and **B**) Assessment of levels of GTP-bound (active) Rac1 GTPase in HMVECs treated with imatinib (10μM), then treated with VEGF (100ng/mL, 2 minutes) or left unstimulated (UT). Rac1-GTP levels, normalized to total Rac1, are quantified in (**B**), relative to levels in vehicle-treated cells (UT). Data are presented as means +/- SD (n=2). (**C**) Evaluation of permeability of HMVECs expressing either control or *Rac1* shRNAs to fluorescein-labeled dextran, following 60 minutes VEGF stimulation with or without imatinib pre-treatment. Data shown are mean fluorescence of samples collected from bottom Transwell chambers, +/- SD of three replicates per treatment. Data are representative of three independent experiments. (**D**) Assessment of Rac1 protein levels following *Rac1* shRNA expression. (*P<0.05; **P<0.01; ***P<0.001).

Similar to Rac1, the Rap1 GTPase has been implicated in the regulation of endothelial barrier integrity by promoting cortical actin deposition and VE-cadherin junctional stabilization [384]. Rap1 activation induces maturation of adherens junctions in unstimulated endothelial cells and inhibits thrombin-induced barrier dysfunction

[385]. The Abl kinases previously have been demonstrated to regulate Rap1 activation in both T cells and epithelial cells, thereby modulating integrin function [291,328]. Interestingly, Abl kinase inhibition led to increased levels of active Rap1 in unstimulated cells, as well as following VEGF treatment (**Figure 4.9A**,**B**). However, imatinib effectively inhibited VEGF-induced endothelial permeability in cells expressing the negative regulator Rap1 GTPase activating protein (Rap1GAP), which prevented both basal and imatinib-induced Rap1 activation (**Figure 4.9C**,**D**). Thus, neither Rac1 nor Rap1 GTPase activation alone account for the anti-permeability effects of imatinib in endothelial cells.

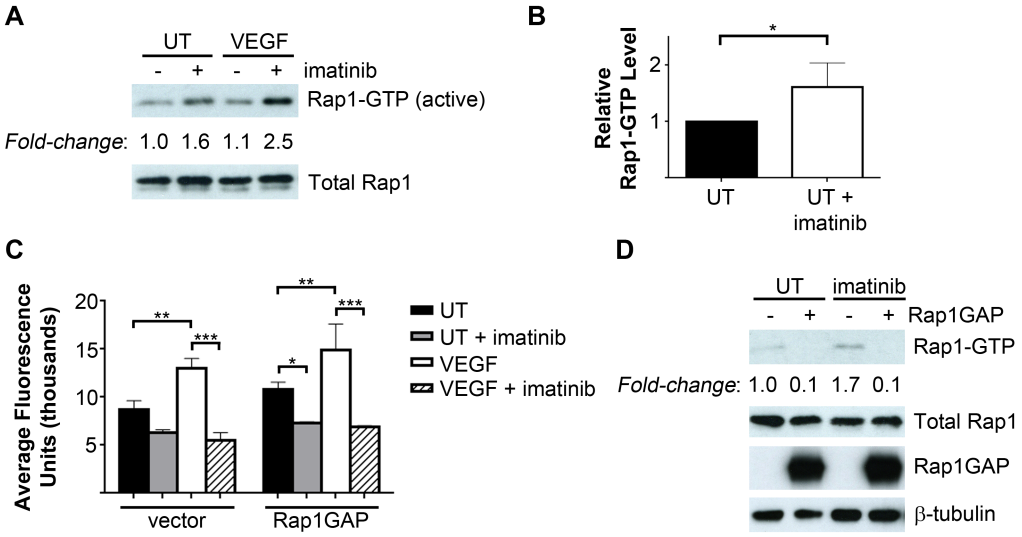

Figure 4.9 Increased Rap1 GTPase Activity Following Abl Kinase Inhibition

(**A** and **B**) Assessment of levels of GTP-bound (active) Rap1 GTPase in HMVECs treated with imatinib (10μM), either treated with VEGF (100ng/mL, 2 minutes) or left unstimulated (UT). Rap1-GTP levels, normalized to total Rap1, are quantified in (**B**), relative to levels in vehicle-treated cells (UT). Data are presented as means +/- SD (n=5). (**C**) Evaluation of permeability of HMVECs expressing either Rap1GAP or vector control

to fluorescein-labeled dextran, following 60 minutes VEGF treatment with or without imatinib pre-treatment. Data shown are mean fluorescence of samples collected from bottom Transwell chambers, +/- SD of three replicates per treatment. Data are representative of two independent experiments. (**D**) Assessment of levels of active, GTP-bound Rap1 in vehicle (UT)- or imatinib-treated cells expressing either Rap1GAP or vector control. (*P<0.05; **P<0.01; ***P<0.001).

4.2.6 Loss of Abl Kinase Activity Impaired Induction of Acto-Myosin Contractility by Endothelial Permeability-Inducing Factors

In addition to the adhesive forces of cell-cell and cell-matrix interactions, the function of the endothelial barrier is modulated by the generation of contractile forces regulated in part by actin-myosin tension [378,379]. A key determinant of acto-myosin contractility is the phosphorylation of the myosin regulatory light chain (MLC2) at serine (S) 19 or diphosphorylation at threonine 18 and S19, which promotes contractility by increasing myosin ATPase activity [98]. Interestingly, while stimulation of HMVECs with VEGF or thrombin increased levels of phospho-MLC2 (S19), the induction of MLC2 phosphorylation by these permeability-inducing factors was decreased by inhibition of Abl kinase activity (**Figure 4.10A**,**C**) or *Abl* knockdown (**Figure 4.10B**). Thus, Abl kinase activity is required for signaling leading to the induction of acto-myosin contractility downstream of endothelial permeability factors.

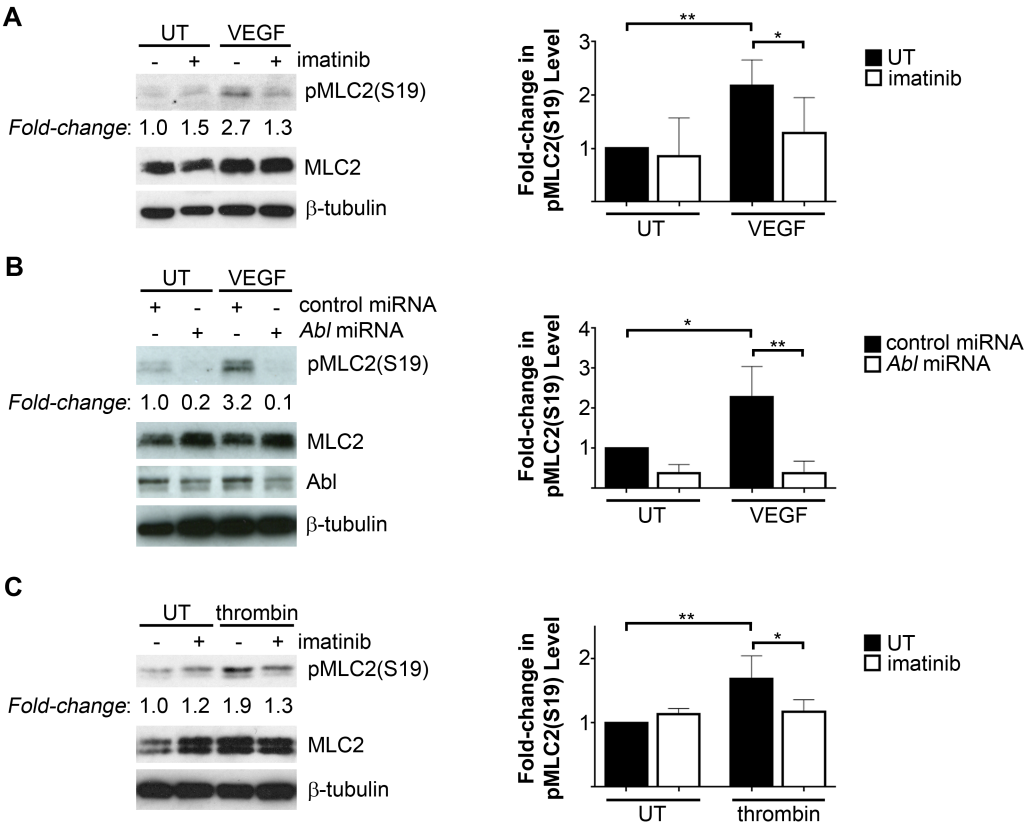

Figure 4.10 Loss of Abl Kinase Activity Impaired MLC2 Phosphorylation in Response to Endothelial Permeability-Inducing Factors

(**A**) Assessment of phospho-MLC2 serine (S) 19 levels in HMVECs following VEGF stimulation (100ng/mL, 5 minutes) with or without imatinib pre-treatment (10μM). pMLC2(S19) levels, normalized to total MLC2, are quantified in the right panel, relative to levels in untreated cells (UT). Data are presented as means +/- SD (n=7). (**B**) Evaluation of pMLC2(S19) levels in HMVECs expressing either control or *Abl* miRNAs following VEGF treatment. pMLC2(S19) levels, normalized to total MLC2, are quantified in the right panel, relative to levels in untreated control miRNA-expressing cells (UT). Data are presented as means +/- SD (n=3). (**C**) Assessment of pMLC2(S19) levels in thrombin-treated HMVECs (1U/mL, 2 minutes), with or without imatinib pre-treatment. pMLC2(S19) levels, normalized to total MLC2, are quantified in the right panel, relative to levels in untreated cells (UT). Data are presented as means +/- SD (n=4). (*P<0.05; **P<0.01).

The phosphorylation status of MLC2 (S19) is regulated by a balance of phosphorylation and dephosphorylation. Phosphorylation of MLC2 in endothelial cells is mediated primarily by the activity of Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK), as well as the Rho GTPase effector Rho kinase (ROCK), while dephosphorylation is mediated by myosin light chain (MLC) phosphatase [30]. ROCK activity additionally increases levels of MLC2 phosphorylation by phosphorylating and inactivating MLC phosphatase [193]. As loss of Abl kinase activity decreased MLC2 (S19) phosphorylation **(Figure 4.10**), we examined whether Abl kinase activity was required for Rho GTPase activation. We did not detect Rho activation following VEGF or histamine treatment (data not shown). However, thrombin stimulation induced potent activation of Rho GTPase, which was not inhibited in imatinib-treated cells (**Figure 4.11**), indicating that the decreased MLC2 phosphorylation observed in imatinib-treated cells was not a result of impaired Rho pathway activation.

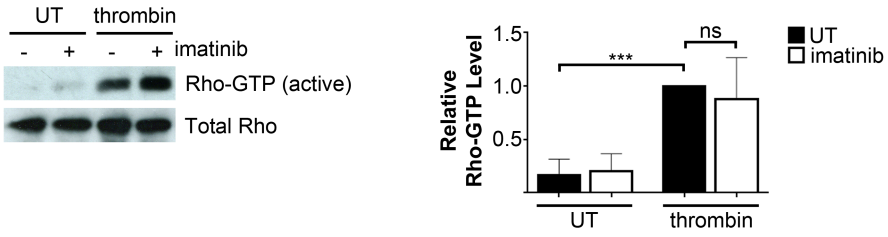

Figure 4.11 Abl Kinase Inhibition Did Not Affect Thrombin-Induced Activation of Rho GTPase

Assessment of levels of GTP-bound (active) Rho GTPase in HMVECs either left unstimulated (UT) or treated with thrombin (1U/mL, 2 minutes), +/- imatinib (10μM). Rho-GTP levels, normalized to total Rho, are quantified in the right panel, relative to

levels in thrombin-stimulated cells. Data are presented as means +/- SD (n=5). (***P<0.001).

4.2.7 Abl Kinase Inhibition Impaired Ca^{2+} Mobilization by Endothelial Permeability-Inducing Factors

The lack of inhibition of thrombin-induced Rho GTPase activation by imatinib suggested that Abl kinases regulate acto-myosin contractility through an alternative pathway. Stimulation of endothelial cells with permeability-inducing agonists is known to increase levels of intracellular calcium, mediated both by release of Ca^{2+} from intracellular stores and by extracellular Ca^{2+} entry through plasma membrane channels, contributing to the activation of Ca^{2+}/calmodulin-regulated enzymes including MLCK [97,386]. Notably, we observed decreased VEGF-induced Ca^{2+} mobilization in the presence of the Abl kinase inhibitors imatinib or GNF-2 (**Figure 4.12A**,**B**). Increases in intracellular Ca^{2+} levels induced by VEGF, thrombin, or histamine were decreased by 30-50% following Abl kinase inhibition (**Figure 4.12C**).

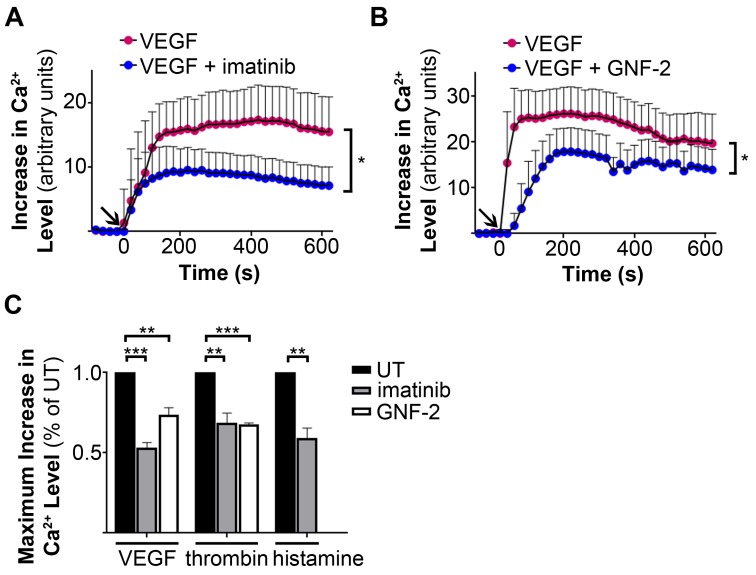

Figure 4.12 Abl Kinase Inhibition Impaired Ca^{2+} Mobilization by Endothelial Permeability-Inducing Factors

(**A** and **B**) Quantification of intracellular Ca^{2+} levels in HMVECs stimulated with VEGF (100ng/mL) with or without (**A**) imatinib (10μM) or (**B**) GNF-2 (15μM) pre-treatment. Values are expressed as increases in intracellular Ca^{2+} levels, relative to levels in unstimulated cells. Arrows indicate timing of addition of permeability-inducing factors. Data are presented as means +/- SD of 35 cells per treatment and are representative of 3 independent experiments. (**C**) Quantification of inhibition of Ca^{2+} mobilization by imatinib or GNF-2. Values shown are maximum intracellular Ca^{2+} levels in HMVECs treated with VEGF, thrombin (1U/mL), or histamine (100μM) in the presence of Abl kinase inhibitors, relative to levels in vehicle-treated cells (UT). Data are presented as means +/- SEM (n=3). (*P<0.05; **P<0.01; ***P<0.001).

The release of Ca^{2+} from intracellular stores is triggered by the binding of inositol-1,4,5-trisphosphate (IP_3) to its cognate receptor on the endoplasmic reticulum (ER) membrane. Cellular IP_3 levels are, in turn, regulated by the activity of phosphoinositide-specific phospholipase C (PLC) family enzymes. In endothelial cells, VEGF-mediated IP_3 generation is regulated by PLCγ activation downstream of VEGF

receptor 2 (VEGFR2) [387]. We observed delayed PLCγ1 activation following VEGF stimulation in HMVECs treated with the Abl kinase inhibitor GNF-2, as assessed by levels of activating phosphorylation of PLCγ1 (Y783) [388] (**Figure 4.13A**,**B**). The levels of phosphorylated PLCγ1 (Y783) induced by VEGF stimulation were reduced by 50% in GNF-2-treated cells 1 minute after VEGF treatment (**Figure 4.13B**). The effect of GNF-2 was transient, as comparable levels of phospho-PLCγ1 (Y783) were detected in vehicle- and GNF-2-treated cells by 5 minutes post-VEGF stimulation. Similar findings were observed upon VEGF stimulation in the presence of imatinib (data not shown). VEGF-mediated PLCγ1 activation requires phosphorylation of VEGFR2 on Y1175 [82]. Interestingly, Abl kinase inhibition also impaired VEGF-induced phosphorylation of VEGFR2 (Y1175) at earlier time points (1 to 2 minutes) (**Figure 4.13A**,**C**). Taken together, these findings suggest that Abl kinase inhibition impairs the VEGF-induced mobilization of intracellular Ca^{2+} in part through decreased VEGF receptor phosphorylation and resulting inhibition of PLCγ activation.

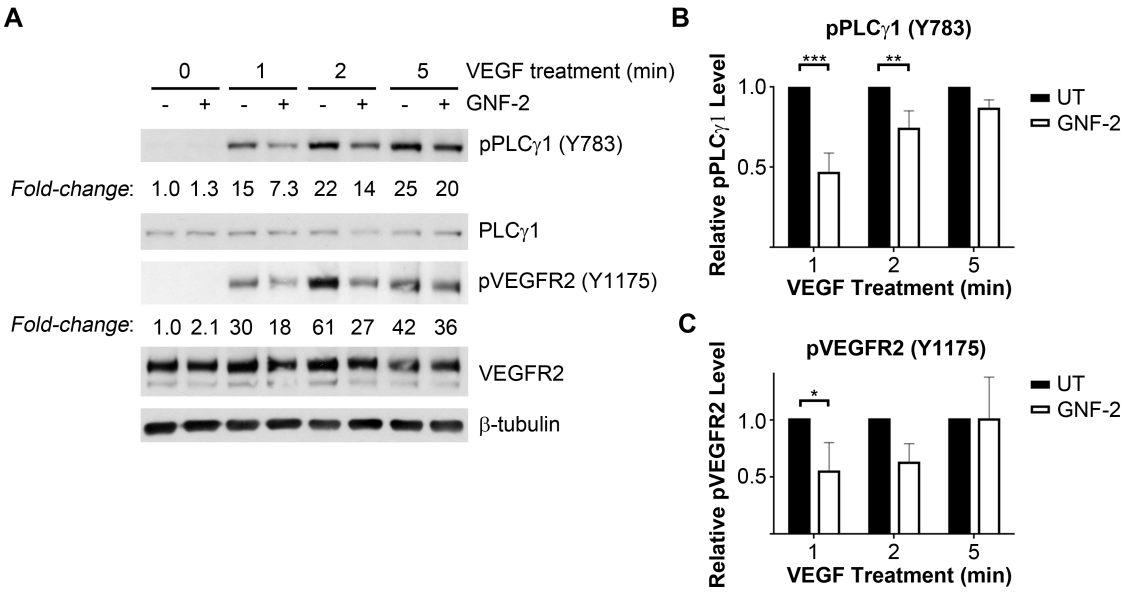

Figure 4.13 Abl Kinase Inhibition Delayed VEGF-Mediated PLCγ Activation

(**A**) Assessment of VEGF-mediated phosphorylation of PLCγ1 and VEGFR2 in HMVECs, with or without GNF-2 pre-treatment. (**B** and **C**) Quantification of levels of (**B**) phospho-PLCγ1 tyrosine (Y) 783 and (**C**) phospho-VEGFR2 Y1175 in HMVECs treated with VEGF +/- GNF-2, relative to levels in vehicle-treated cells (UT) at each time point. Data are presented as means +/- SD (n=3). (*P<0.05; **P<0.01; ***P<0.001).

4.3 Discussion

Excessive vascular leakage is a feature of a wide range of pathological conditions, leading to complications including edema and increased tissue damage following ischemic stroke and myocardial infarction, increased interstitial fluid pressure in cancers, and pulmonary dysfunction in acute respiratory distress syndrome [31,32]. Efforts to prevent this increased vascular permeability are complicated, in part, by the multiple permeability-inducing factors involved in these disorders [389]. In the current

study, we have identified the Abl family kinases, Abl and Arg, as mediators of endothelial barrier dysfunction induced by several disparate permeability factors, including agonists signaling through both receptor tyrosine kinases (VEGF) and G protein-coupled receptors (thrombin and histamine). Importantly, Abl kinase inhibition, using either imatinib or the Abl/Arg-specific allosteric inhibitor GNF-2, impaired VEGF-induced vascular permeability both in cultured endothelial cells and in mice. We showed for the first time a direct requirement for Abl in VEGF-induced dermal vascular leakage in mice lacking endothelial *Abl* expression. Loss of Abl kinase activity protects against endothelial barrier dysfunction; this effect is accompanied by activation of the barrier-stabilizing GTPases Rac1 and Rap1, as well as inhibition of both agonist-induced Ca^{2+} mobilization and generation of acto-myosin contractility. A model for the proposed role of the Abl kinases in signaling pathways regulating endothelial permeability is shown in **Figure 4.14**.

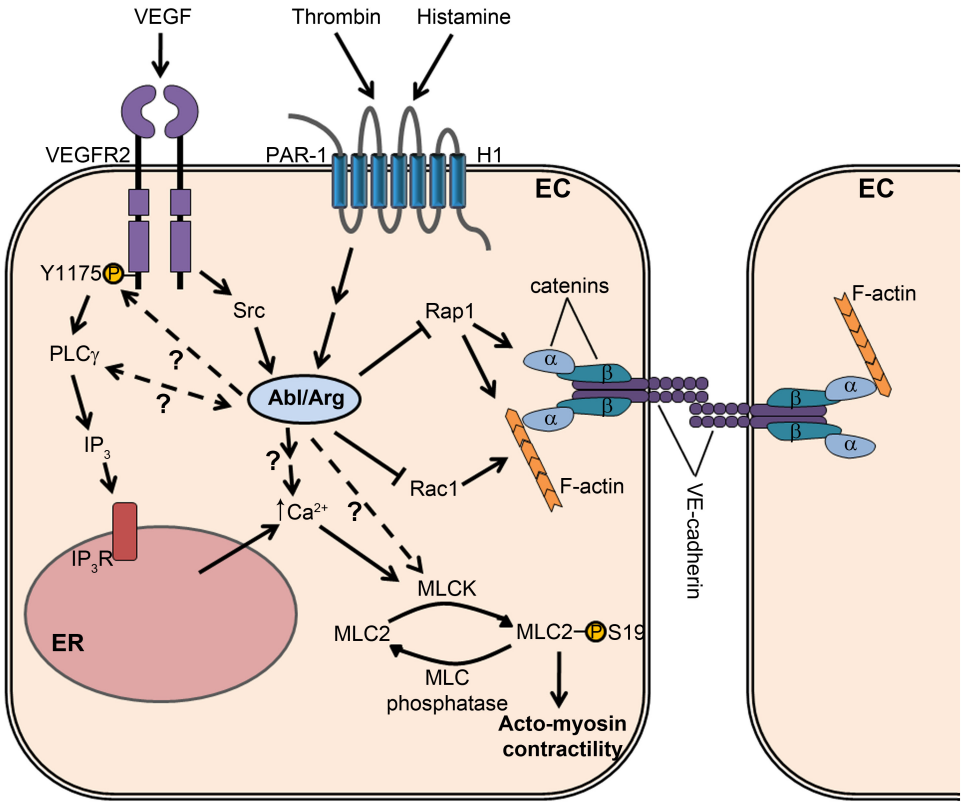

Figure 4.14 Model for the Role of the Abl Kinases in Signaling Pathways Regulating Endothelial Permeability

The Abl and Arg kinases are activated in endothelial cells downstream of receptors for the permeability-inducing factors VEGF, thrombin, and histamine. VEGF-mediated Abl kinase activation requires Src family kinase activity. The Abl kinases positively regulate phosphorylation of MLC2 (S19) in response to these permeability-inducing agonists, likely through regulating the activity of Ca^{2+}/calmodulin-dependent targets such as MLCK. Abl kinase activity is required for maximal Ca^{2+} mobilization in response to stimulation with permeability-inducing factors. The Abl kinases additionally modulate VEGF-induced phosphorylation of VEGFR2 at Y1175, which regulates downstream PLCγ activation, IP_3 generation, and ER Ca^{2+} release. Abl kinases promote Ca^{2+} mobilization by thrombin and histamine by mechanisms yet to be characterized. Abl kinases negatively regulate basal activity levels of the Rac1 and Rap1 GTPases, which have been shown to support endothelial barrier function by promoting cortical actin deposition and adherens junction stability. *Abbreviations*: EC, endothelial cell; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; Y, tyrosine; PAR-1, protease-activated receptor 1 (thrombin); H1, histamine H1 receptor; MLC2, myosin

regulatory light chain; S, serine; MLCK, myosin light chain kinase; PLC, phospholipase C; IP_3, inositol-1,4,5-trisphosphate; IP_3R, IP_3 receptor; ER, endoplasmic reticulum.

The endothelial barrier is regulated by a dynamic balance of adhesive and contractile forces [30]. We found that inhibition of the Abl kinases led to increased activation of the endothelial barrier-promoting GTPases Rac1 and Rap1, which promote cortical actin remodeling and adherens junction stability [190,191,192,384,385]. The Abl kinases previously have been linked either to positive or negative regulation of GTPase activation, depending upon the cellular context. The Abl kinases are required for Rac1 activation downstream of cadherin engagement in epithelial cells [294], as well as Rap1 activation following T cell receptor engagement or chemokine stimulation in T cells [291,292]. In contrast, expression of constitutively-active Abl kinases reduced levels of active Rap1 in epithelial and HEK293 cells, as a result of Abl-mediated tyrosine phosphorylation of the CrkII adaptor and disruption of the association between CrkII and the Rap1 guanine nucleotide exchange factor (GEF) C3G [328,390]. It remains to be determined whether the enhanced Rap1 activation we observed upon Abl kinase inhibition in endothelial cells results from increased CrkII/C3G interaction and C3G GEF activity. However, our findings that reduced Rac1 expression or Rap1 activity did not prevent imatinib-mediated endothelial barrier stabilization suggest that Rac1- and Rap1-independent pathways mediate the anti-permeability effects of Abl/Arg kinase inhibition.

Notably, inhibition of Abl kinase activity impaired VEGF- and thrombin-induced phosphorylation of the myosin regulatory light chain (MLC2), which regulates myosin ATPase activity and induction of barrier-destabilizing acto-myosin contractility [98]. However, Abl kinase inhibition in endothelial cells did not affect activation of the Rho/ROCK pathway, which increases MLC2 phosphorylation through inhibitory phosphorylation of myosin light chain phosphatase [193]. In contrast, in fibroblasts, the Arg kinase previously has been linked to inhibition of acto-myosin contractility during integrin-mediated adhesion and migration, through phosphorylation and activation of the RhoA inhibitor p190RhoGAP [323]. Similarly, Abl kinase inhibition in epithelial cells leads to increased baseline Rho activation, formation of actin stress fibers, and weakened intercellular adhesion [294]. These disparate findings may be explained by differential roles for Abl kinases in the regulation of signaling pathways mediating basal versus agonist-induced Rho activation and acto-myosin contractility in various cell types.

Phosphorylation of the myosin regulatory light chain can be regulated by the Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) [97]. Interestingly, treatment with the endothelial barrier-promoting bioactive lipid sphingosine-1-phosphate was shown to increase Abl-mediated MLCK (Y464) phosphorylation [346], raising the possibility that Abl kinase activation by endothelial permeability-inducing mediators similarly may contribute to MLCK phosphorylation and activation. Tyrosine phosphorylation of MLCK has been linked to enhanced enzymatic activity at lower Ca^{2+}

concentrations [391]. Notably, we found that increases in intracellular Ca^{2+} levels induced by endothelial barrier-disrupting factors were attenuated by Abl kinase inhibition. Thus, we postulate that decreased intracellular Ca^{2+} mobilization in cells with reduced Abl kinase activity might contribute to impaired agonist-stimulated MLCK activity, leading to the observed reduction in MLC2 phosphorylation. Further investigation is needed to characterize fully the effects of Abl kinase inhibition on MLCK activity, as well as to determine whether the impaired Ca^{2+} mobilization inhibits activation of other Ca^{2+}-regulated enzymes involved in endothelial barrier dysfunction, such as PKCα [387].

Consistent with the observed impairment of agonist-mediated Ca^{2+} mobilization, we found that VEGF-induced PLCγ activation was delayed in the absence of Abl kinase activity. Interestingly, phosphorylation of VEGFR2 (Y1175), which is required for PLCγ activation [82], similarly was delayed in endothelial cells treated with Abl kinase inhibitors. Previous work has demonstrated Abl kinase-mediated phosphorylation of the PDGF and epidermal growth factor receptors [260,392]; however, it remains to be determined whether VEGFR2 is an Abl kinase target. In addition, we have previously identified a bi-directional link between PLCγ and Abl in PDGF-stimulated fibroblasts, whereby PLCγ is required for Abl kinase activation, and in turn, Abl modulates PLCγ enzymatic activity [286]. Further investigation will be required to determine if a similar PLCγ-Abl connection exists in VEGF-stimulated endothelial cells. In all, these findings

suggest that impaired VEGF-induced Ca^{2+} mobilization upon Abl kinase inhibition may result, at least in part, from decreased PLCγ-mediated IP_3 generation, resulting in impaired release of endoplasmic reticulum (ER) Ca^{2+} stores. However, distinct mechanisms are likely to be involved in the inhibition of thrombin- and histamine-induced Ca^{2+} mobilization, which is regulated by G protein-mediated activation of PLCβ [387]. In this regard, previous studies have suggested a role for tyrosine kinases in regulating extracellular calcium entry through plasma membrane channels following depletion of intracellular Ca^{2+} stores (store-operated Ca^{2+} influx) [387].

In summary, we have demonstrated a requirement for the Abl kinases in induction of endothelial permeability by VEGF and the inflammatory mediators thrombin and histamine. Our findings and recent reports suggest that the endothelial barrier-protective effects of Abl kinase inhibition result from several distinct mechanisms, including promoting cell-cell and cell-matrix adhesion [345], as well as impairing induction of acto-myosin contractility. While the precise Abl kinase targets involved in the regulation of these pathways remain to be characterized, the existence of multiple pathways mediating the anti-permeability effects of Abl kinase inhibition suggests that pharmacological targeting of the Abl kinases may be capable of inhibiting endothelial permeability induced by a broad range of agonists. However, further studies will be needed to evaluate the involvement of the Abl kinases in endothelial barrier dysfunction mediated by additional permeability-inducing factors, as well as to

determine whether *in vivo* pharmacological or genetic inactivation of the Abl kinases may have protective effects in disorders involving pathological vascular leakage.

5. Discussion and Future Directions

Proper endothelial cell function is required for maintenance of normal cardiovascular physiology, ensuring appropriate regulation of vascular barrier function and tissue perfusion [2]. These functions are regulated in part by a variety of soluble mediators, which signal through endothelial cell surface receptors to influence processes including proliferation, survival, migration, and vascular stability [64,65]. The work described in the preceding chapters supports an important role for the Abl family of non-receptor tyrosine kinases (Abl and Arg) in endothelial cell responses to several pro-angiogenic and permeability-inducing factors, including the pro-survival effects of VEGF, bFGF, and Angpt1 (Chapter 3) and the destabilization of the endothelial barrier in response to VEGF, thrombin, and histamine (Chapter 4). Most notably, these studies have shown that the Abl family kinases play a crucial role in vascular function *in vivo*, as mice lacking endothelial expression of the Abl/Arg kinases died at late embryonic and perinatal stages of development (Chapter 3).

5.1 Role of the Abl Kinases in Vascular Function

Previous studies have reported cardiotoxic effects (left ventricular dysfunction; heart failure) [2,350,351,352] as well as vaso-occlusive events [354,355,356] in a subset of cancer patients following long-term treatment with Abl kinase inhibitors. The work detailed in Chapter 3 has demonstrated, using a mouse genetic model of endothelial *Abl* inactivation, that expression of Abl/Arg kinases is required for proper vascular function

during embryonic development. *Abl*^{ECKO}; *Arg*$^{-/-}$ mice died at late embryonic stages of development or shortly after birth and displayed localized hepatic vascular loss and tissue damage (Chapter 3). These vascular defects may result from impaired endothelial cell survival, as we have demonstrated that loss of Abl kinase function leads to increased susceptibility to endothelial cell apoptosis both *in vitro* and *in vivo*.

5.1.1 Variability of Cardiovascular Phenotypes of *Abl*^{ECKO}; *Arg*$^{+/-}$ Mice – Effects of Genetic Background?

In contrast to the late-stage embryonic and perinatal lethality of *Abl*^{ECKO}; *Arg*$^{-/-}$ mice, endothelial *Abl*-knockout mice on an *Arg*$^{+/-}$ background (*Abl*^{ECKO}; *Arg*$^{+/-}$) were viable, indicating that the remaining *Arg* allele largely compensates for the loss of endothelial *Abl* expression. Interestingly, a subset (approximately 15%) of these *Abl*^{ECKO}; *Arg*$^{+/-}$ mice displayed dramatic cardiovascular phenotypes, including left ventricular scarring, lung fibrosis, and right ventricular hypertrophy. This phenotypic heterogeneity did not result from differing degrees of *Abl* inactivation, as a significant decrease in endothelial *Abl* expression was observed even in *Abl*^{ECKO}; *Arg*$^{+/-}$ mice that did not exhibit overt cardiovascular pathology (**Figure 5.1A**,**B**). This finding suggests that loss of endothelial *Abl* expression predisposes these mice to vascular complications, but that additional factors are required for overt pathological cardiovascular phenotypes.

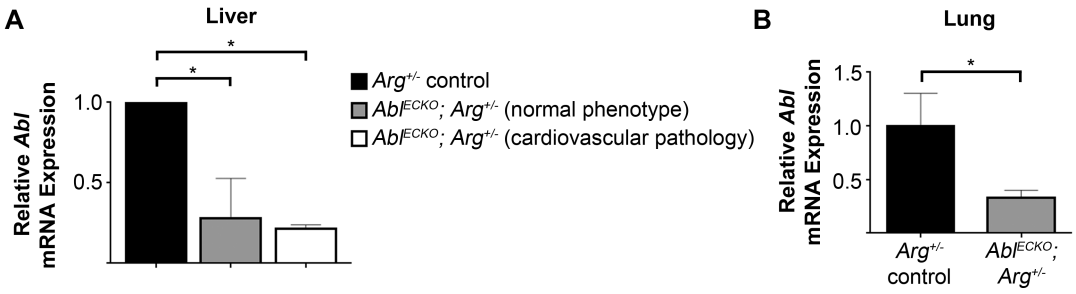

Figure 5.1 Decreased *Abl* mRNA Expression in *Abl*^{ECKO}; *Arg*$^{+/-}$ Mice, Regardless of Cardiovascular Phenotype

Assessment of *Abl* knockdown levels in *Abl*^{ECKO}; *Arg*$^{+/-}$ mice. (**A**) Endothelial cells (CD105^{+}/CD45^{-}/CD31^{+}) were isolated from the livers of adult *Arg*$^{+/-}$ control mice and 2 subsets of *Abl*^{ECKO}; *Arg*$^{+/-}$ mice: (1) phenotypically normal *Abl*^{ECKO}; *Arg*$^{+/-}$ mice and (2) *Abl*^{ECKO}; *Arg*$^{+/-}$ mice which exhibited cardiovascular pathology. Real-time RT-PCR analysis of *Abl* mRNA expression demonstrated decreased *Abl* levels in *Abl*^{ECKO}; *Arg*$^{+/-}$ mice, regardless of cardiovascular phenotype. Data are shown as means +/- SD, normalized to *Abl* expression in *Arg*$^{+/-}$ control mice (n=2 mice per genotype). (**B**) *Abl* mRNA expression was assessed in endothelial cells isolated from the lungs of adult *Arg*$^{+/-}$ control mice and phenotypically normal *Abl*^{ECKO}; *Arg*$^{+/-}$ mice (n=3 mice per genotype, pooled). Data are presented as means +/- SD of 3 replicates, normalized to *Abl* expression in *Arg*$^{+/-}$ control mice. (*P<0.05).

The variability of the phenotype of *Abl*^{ECKO}; *Arg*$^{+/-}$ mice is similar to the incomplete penetrance of the immunological phenotypes observed in global *Abl* knockout mice [330,331]. It is possible that genetic modifiers on a mixed (129/SvJ x C57BL/6) genetic background may underlie the different degrees of cardiovascular phenotype observed; indeed, global Abl deletion leads to different cardiac phenotypes, depending upon the mouse genetic background [349]. Notably, Koleske and colleagues independently generated *Abl*$^{flox/flox}$; *Arg*$^{-/-}$; *Tie2-Cre*$^{+}$ mice to examine the role of the Abl kinases in macrophage function during parasitic infection [393]. In contrast to the

embryonic and perinatal lethality of our *Abl*^{ECKO}; *Arg*$^{-/-}$ mice, the mice generated in that study apparently were viable, although their vascular phenotype was not analyzed. It is possible that differences in genetic background may contribute to these contrasting phenotypes, as the Koleske group's mice were in a mixed genetic background (129Sv/J x C57BL/6), whereas our *Abl*^{ECKO}; *Arg*$^{-/-}$ mice were backcrossed six generations into the C57BL/6 background [393].

5.1.2 Localized, Sporadic Vascular Loss in *Abl*^{ECKO} Mice

Loss of endothelial expression of the Abl kinases did not affect overall vascular density, branching, and patterning, indicating that the Abl kinases are not required for angiogenic remodeling after E9.5 (the stage at which *Tie2-Cre*-mediated recombination has occurred in the majority of endothelial cells, [367]). Rather, loss of endothelial Abl/Arg kinases likely adversely affects vascular maintenance and stability, rather than vessel formation. However, it remains possible that subtle structural defects during vessel formation could contribute to the phenotypes observed later in development in mutant embryos. Electron microscopy analysis of mid-gestation *Abl*^{ECKO}; *Arg*$^{-/-}$ embryos would be required for the detailed examination of endothelial structure in these mice. It similarly would be of interest to examine the effects of inducible endothelial *Abl* inactivation (i.e., by crossing to *VE-cadherin-CreER*^{T2}, *Tie2-CreER*^{T2}, or *Pdgfb-iCreER* mice [394,395,396,397]) on vascular structure and function at later stages of embryonic and postnatal development.

The primary phenotype observed in mice lacking endothelial Abl kinase expression was a localized loss of blood vessels, which led to subsequent tissue infarction and organ damage. The focal and sporadic nature of this vascular loss was striking and, in combination with our observation that loss of the Abl kinases sensitizes endothelial cells to stress-induced apoptosis *in vitro*, leads us to speculate that the vascular damage observed in these mice may result from localized endothelial cell apoptosis in response to cumulative vascular stresses in the absence of the Abl kinases. The sporadic vascular occlusions observed in some adult Abl^{ECKO}; $Arg^{+/-}$ mice may result from endothelial cell apoptosis and vascular damage, which may contribute to thrombosis and defective tissue perfusion. Interestingly, while adult Abl^{ECKO}; $Arg^{+/-}$ mice exhibited leukocyte and red blood cell counts comparable to $Arg^{+/-}$ control mice, these mice showed a 10% reduction in the number of circulating platelets (**Table 5.1**), suggesting that thrombosis in these mice may contribute to a slight depletion of circulating platelets. However, as *Tie2-Cre*-mediated recombination also inactivates *Abl* in hematopoietic cells (including megakaryocytes) [363], we cannot exclude the possibility that Abl kinase activity in megakaryocytes or platelets may regulate platelet generation or function. In this regard, treatment of mice with the ATP-competitive dual Src/Abl kinase inhibitor dasatinib leads to mild thrombocytopenia as a result of inhibition of platelet formation [398].

Table 5.1 Complete Blood Counts (CBC) Analysis of *Abl*^{ECKO}; *Arg*$^{+/-}$ Adult Mice

CBC Analysis Parameter	***Arg*$^{+/-}$ control** (n=8)	***Abl*^{ECKO}; *Arg*$^{+/-}$** (n=8)
White Blood Cells		
Total WBC (K/μL)	14.02 +/- 4.92	12.77 +/- 3.27
Lymphocytes (K/μL)	10.09 +/- 5.01	9.76 +/- 3.91
Neutrophils (K/μL)	3.27 +/- 1.71	2.40 +/- 1.32
Monocytes (K/μL)	0.63 +/- 0.50	0.58 +/- 0.17
Eosinophils (K/μL)	0.022 +/- 0.029	0.007 +/- 0.004
Basophils (K/μL)	0.027 +/- 0.037	0.019 +/- 0.013
Red Blood Cells/ Hematocrit		
RBC (M/μL)	9.30 +/- 0.85	9.61 +/- 0.46
Hematocrit (%)	45.21 +/- 3.74	46.60 +/- 1.72
Platelets		
Platelets (K/μL)	1295.8 +/- 118.2	1166.4 +/- 81.0 *
Mean Platelet Volume (fL)	4.50 +/- 0.26	4.49 +/- 0.20

Blood samples were collected from 3- to 4-month-old female *Abl*^{ECKO}; *Arg*$^{+/-}$ mice (phenotypically normal) and age/sex-matched *Arg*$^{+/-}$ control mice via cardiac puncture. EDTA-treated blood samples were used for analysis of complete blood counts (CBC). CBC analysis was performed by the Duke University School of Medicine Division of Laboratory Animal Resources (DLAR) Veterinary Diagnostic Lab (VDL), using an Abbot Cell Dyn 3700 analyzer. Data are presented as means +/- SD. (*P<0.05). Abbreviations: WBC, white blood cells; RBC, red blood cells; K, x10^3; M, x10^6.

Endothelial cell death or damage has been reported in numerous vascular pathologies [371], and a resulting loss of endothelial integrity can trigger coagulation by exposing thrombogenic ECM components to the circulation [399,400]. Endothelial cells also typically synthesize a variety of both soluble and membrane-bound factors (nitric oxide, prostacyclin, tissue factor pathway inhibitor, thrombomodulin, etc.) which function to maintain the vasculature in an anti-thrombotic state [401]; thus, endothelial cell damage or dysfunction can contribute to thrombosis through altered production of these regulatory factors. It is possible that the sporadic thrombosis observed in mice lacking endothelial Abl kinase expression may result from a specific defect in endothelial regulation of coagulation and hemostasis, in addition to increased susceptibility to endothelial cell apoptosis. Interestingly, Abl kinase activity is required for LPS-induced nitric oxide (NO) production in macrophages (Greuber and Pendergast, unpublished, and [402]); however, it is not known whether the Abl kinases may play a similar role in the regulation of endothelial cell NO production. Thus, it may be of interest to examine whether endothelial *Abl* inactivation affects thrombus formation or resolution in murine thrombosis models [403], particularly in light of vascular occlusive events observed in some nilotinib-treated cancer patients [354,355].

5.2 Role of the Abl Kinases in Angpt/Tie2 Signaling

Endothelial *Abl* inactivation in mice resulted in sporadic thrombosis, endothelial cell apoptosis, and vascular loss. Notably, localized thrombosis and endothelial cell

apoptosis similarly were observed upon endothelial *Vegf* deletion, which was attributed to a requirement for intracrine VEGF signaling for endothelial cell survival and maintenance of vascular homeostasis [71]. Similarly, a role for Tie2 signaling in maintaining quiescence and homeostasis in the adult vasculature has been proposed, which correlates with the detection of Tie2 phosphorylation/activation in the vasculature of all adult tissues examined [162]. Our current studies have uncovered a novel role for the Abl kinases in Angpt1/Tie2 signaling, as loss of Abl/Arg kinase expression resulted in decreased Tie2 receptor expression, as well as diminished Angpt1-mediated signaling responses and pro-survival effects. The Abl kinases appear to play a dual role in Angpt1/Tie2 signaling through the regulation of Tie2 expression and modulation of signaling networks required for Angpt1-mediated endothelial cell survival.

5.2.1 Altered Expression of Tie2 and Angiopoietins Following *Abl*/*Arg* Knockdown

Interestingly, in addition to Tie2 receptor downregulation, *Abl*/*Arg* knockdown led to altered expression of its angiopoietin ligands. While mRNA and protein levels of the antagonistic Angpt2 ligand were increased following *Abl*/*Arg* depletion, expression of the Tie2 agonist Angpt1 was decreased. However, changes in Angpt1 and Angpt2 expression occurred 72 to 96 hours after *Abl*/*Arg* knockdown, while Tie2 expression was decreased at earlier time points (within 24 to 48 hours), suggesting that altered angiopoietin levels may be secondary effects of *Abl*/*Arg* depletion, induced by Tie2 downregulation. The mechanisms by which loss of Abl kinase expression contributes to

changes in Tie2 and angiopoietin expression remain to be determined. Reporter assays (i.e., Tie2 promoter-driven luciferase expression, [404]) will shed light on whether the observed changes in gene expression result from altered transcription, rather than effects on mRNA stability or miRNA-mediated regulation, and may identify promoter regions important for this regulation. *Tie2* expression is promoted by transcription factors including the Ets family member NERF2 (new Ets-related factor 2) [404], as well as Kruppel-like factor 2 (KLF2) [405], and *Tie2* levels are increased in response to the proinflammatory cytokines TNFα and interleukin-1β (IL-1β) [406]. In contrast, decreased *Tie2* mRNA levels have been observed in response to a variety of cellular stresses, including endoplasmic reticulum (ER) stress [407] and exposure to high glucose levels [408]. Tie2 expression also may be reduced in response to hypoxia [138], although other studies have shown hypoxia-induced Tie2 upregulation [406]. Thus, the decrease in *Tie2* expression observed following loss of the Abl kinases may result either from direct effects on transcription factor activity or indirectly through induction of cellular stress. Notably, imatinib treatment of cardiomyocytes has been linked to induction of ER stress [350], although it remains to be determined if loss of Abl kinase function has similar effects in endothelial cells.

In contrast to Tie2, which is broadly expressed in endothelial cells in the adult vasculature [162], Angpt2 expression is not readily detectable in quiescent vessels in adult tissues [134]. However, increased Angpt2 levels are observed at sites of

inflammation or angiogenic remodeling [135], and *Angpt2* mRNA expression is upregulated in response to hypoxia or stimulation with cytokines or growth factors including VEGF [138,139]. *Angpt2* expression is promoted by Ets family transcription factors [409], as well as the forkhead transcription factor FOXO1 (also known as FKHR) [176]. Interestingly, Angpt1-mediated Akt activation leads to phosphorylation and inactivation of FOXO1, which results in suppression of *Angpt2* expression [176], suggesting the presence of a regulatory loop controlling angiopoietin expression and Angpt/Tie2 pathway activity. PI3K/Akt pathway activation by Angpt1 regulates both *Tie2* and *Angpt2* expression through induction of KLF2 expression [179,410]. In addition to promoting *Tie2* expression, KLF2 suppresses expression of *Angpt2* [405]. Thus, it is possible that the increased *Angpt2* levels observed following *Abl*/*Arg* depletion may be a consequence of diminished Angpt1/Tie2-mediated signaling. It would be of interest to determine whether *Tie2* knockdown would result in similar changes in angiopoietin expression to those detected after knockdown of *Abl*/*Arg*. It also is unclear whether the observed increase in endothelial cell Angpt2 levels may affect Tie2 signaling or downstream responses, as Angpt2 expression has been linked to both protective and destabilizing effects in different cellular contexts [151,196].

5.2.2 Role of the Abl Kinases in Angiopoietin-1/Tie2 Signaling

Importantly, in addition to reducing Tie2 levels, *Abl*/*Arg* knockdown resulted in decreased Angpt1/Tie2 signaling responses (particularly Akt phosphorylation), as well

as diminished Angpt1-mediated pro-survival effects. However, re-expression of exogenous Tie2 did not fully restore the anti-apoptotic effects of Angpt1, suggesting that the Abl kinases also modulate Angpt1-mediated endothelial cell survival through mechanisms other than regulation of Tie2 expression. Interestingly, we found that Angpt1 stimulation of endothelial cells leads to activation of the Abl kinases. Thus, the Abl kinases additionally may play a role in signaling responses downstream of the Tie2 receptor. As Angpt1 can bind to integrins as well as the Tie2 receptor, it is possible that Abl kinase activation may also occur downstream of Angpt1/integrin binding [154]. Analysis of Angpt1-mediated Abl kinase activation in *Tie2*-knockdown endothelial cells will be necessary to examine this possibility.

Further studies will be required to determine how the Abl kinases are activated following Angpt1/Tie2 binding, as well as to investigate the involvement of these kinases in mediating Angpt1/Tie2 downstream cellular responses. It is possible that the Abl kinases are recruited to the Tie2 receptor following Angpt1 stimulation either directly (as has been observed following activation of the PDGF receptor [260]) or indirectly through interaction with adaptor or docking proteins including Grb2 or Dok-R [264,411]. While the data presented here suggest that the Abl kinases may function downstream of Tie2 to mediate endothelial cell survival, it will be of interest to determine whether Abl/Arg may modulate other endothelial cell responses to Angpt1, including migration, as well as anti-permeability and anti-inflammatory effects. In this

regard, several signaling proteins required for Angpt1/Tie2-dependent endothelial cell migration, including Dok-R, Nck and PAK [183,184], are binding partners and/or substrates of the Abl kinases [264,411,412].

Interestingly, *Abl*/*Arg* depletion led to a more pronounced inhibition of Angpt1-mediated Akt phosphorylation than of Erk activation. The activation of distinct Tie2 complexes either at cell-cell or cell-matrix contacts has been linked to preferential activation of the Akt or Erk pathways, respectively [179,180]. Thus, an intriguing possibility is that the Abl kinases may modulate Tie2 signaling responses particularly at cell-cell contacts. Alternatively, the Abl kinases may have a role in receptor trafficking, including Angpt1-induced translocation of Tie2 to cell-cell contacts in confluent endothelial cells. In this regard, the Abl kinase has been shown to regulate EGF receptor endocytosis [392] and is required for proper Notch receptor endocytic trafficking [413]. In addition, single knockdown of either *Abl* or *Arg* demonstrated that loss of either kinase was sufficient to inhibit Angpt1-mediated signaling. However, in contrast to dual *Abl*/*Arg* knockdown, *Abl* depletion alone did not decrease total Tie2 receptor levels, although Angpt1-induced Tie2 phosphorylation was diminished (**Figure 3.20B**). It will be of interest to determine whether Tie2 localization is altered upon *Abl* knockdown, as well as to examine whether the Abl and Arg kinases may differentially regulate Angpt1/Tie2-mediated signaling through effects on Tie2 activation and expression, respectively.

5.2.3 Effects of Abl Kinase Inhibition on the Angiopoietin/Tie2 Pathway

Our studies evaluating the effects of loss of *Abl*/*Arg* kinase expression on angiopoietin/Tie2 expression and signaling have suggested that the observed decreases in Tie2 expression and Angpt1-mediated endothelial cell survival may result from loss of the tyrosine kinase activity and/or scaffolding function of the Abl family kinases. To evaluate the contribution of Abl kinase activity to regulation of the angiopoietin/Tie2 pathway, we examined the effects of pharmacological inhibition of the Abl kinases on angiopoietin/Tie2 expression and Angpt1-mediated endothelial cell survival. Treatment with the Abl family kinase inhibitor imatinib (10μM) resulted in substantial inhibition of Angpt1-mediated HUVEC survival following serum-starvation (**Figure 5.2A**). A slight reduction in Tie2 protein levels was observed after 24 hours imatinib treatment, along with a more pronounced increase in Angpt2 levels (**Figure 5.2B**). A similar increase in Angpt2 protein expression occurred after Abl kinase inhibition with nilotinib (**Figure 5.2C**), as well as treatment with a lower concentration of imatinib (5μM). However, no change in Tie2 expression was observed after nilotinib or 5μM imatinib treatment (**Figure 5.2C**). Thus, it is possible that the reduced Tie2 levels observed following treatment with 10μM imatinib may be due to inhibition of multiple kinases that are targeted by higher imatinib levels. Importantly, the increase in Angpt2 levels observed following imatinib treatment was reduced by expression of imatinib-resistant Abl and Arg mutants (Abl-T315I, [414]) (**Figure 5.2D**), demonstrating that imatinib-mediated

Angpt2 upregulation likely results from inhibition of Abl/Arg. It remains to be determined whether Abl kinase inhibition similarly increases Angpt2 levels *in vivo*, as well as the physiological effects of this increase in Angpt2 expression in cells lacking active Abl kinases.

Interestingly, an increased ratio of Angpt2 to Angpt1 expression has been reported in a number of disorders involving vascular dysfunction, including diabetes, hyperoxic lung injury, and sepsis [202,203,204,205]. Further, elevated circulating Angpt2 levels have been correlated with disease progression in sepsis [135]. If Abl kinase inhibition also leads to increased Angpt2 expression *in vivo*, it is possible that use of Abl kinase inhibitors could have deleterious effects on progression of these disorders. On the other hand, increased Angpt2 expression has been proposed to serve a protective function in stressed endothelial cells (following inhibition of pro-survival PI3K/Akt signaling) [151]. Thus, an alternative interpretation of our findings is that the increase in Angpt2 levels is a component of an endothelial cell stress response following Abl kinase inhibition. Further study will be needed to investigate these possibilities.

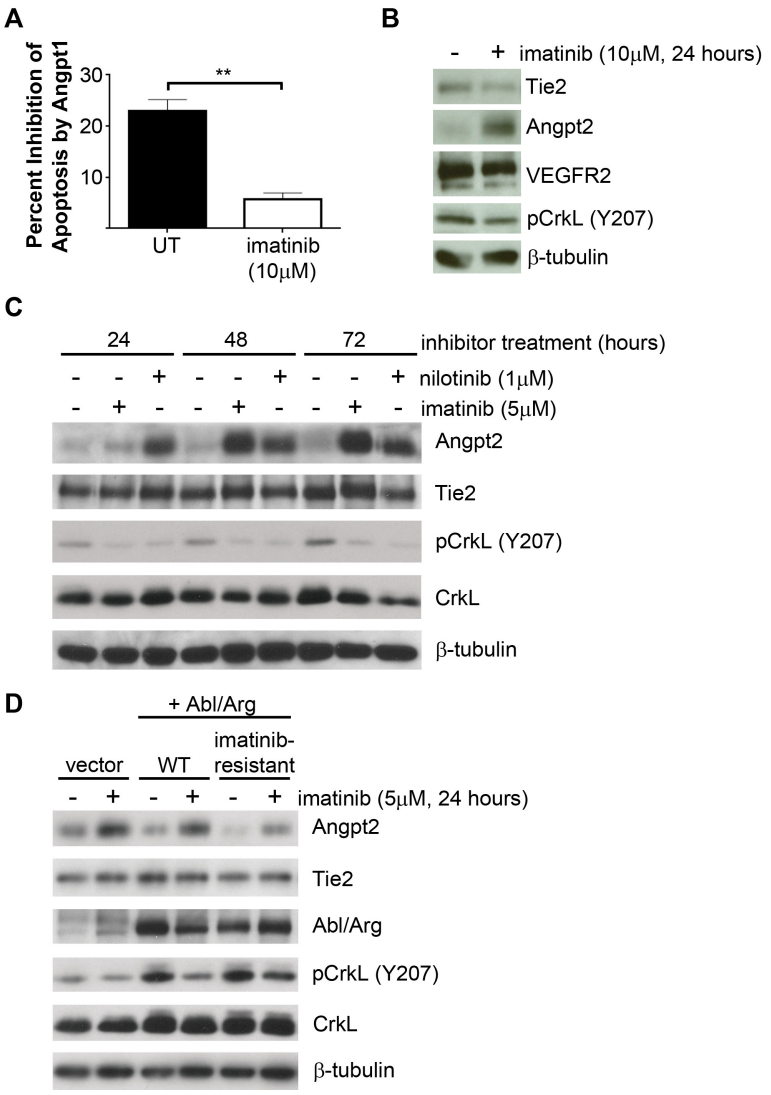

Figure 5.2 Inhibition of Angpt1-Mediated Endothelial Cell Survival and Altered Angiopoietin/Tie2 Expression Following Pharmacological Inhibition of the Abl Kinases

(**A**) Analysis of levels of apoptosis in HUVECs following 24 hours serum-starvation in the presence of Angpt1 (200ng/mL) +/- imatinib (10μM). Values are expressed as percent inhibition of apoptosis by Angpt1 relative to serum-starved (non-supplemented) cells. Data are presented as means +/- SEM (n=3; **P<0.01). (**B**) Assessment of Angpt2 and Tie2 protein levels in HUVECs treated with 10μM imatinib for 24 hours. (**C**) Analysis of Angpt2 and Tie2 protein expression in HUVECs after treatment with Abl family kinase inhibitors (5μM imatinib or 1μM nilotinib) for 24, 48, or 72 hours (culture medium with

inhibitors was changed daily). (**D**) Assessment of Angpt2 and Tie2 protein levels in HUVECs infected with retroviruses expressing wild-type murine Abl and Arg proteins or imatinib-resistant Abl and Arg proteins (mAbl-T315I and analogous mArg mutation; numbering indicates position of mutated threonine residue in mAbl 1a isoform) after imatinib treatment (5μM, 24 hours). All results are representative of at least 2 independent experiments.

5.3 Role of the Abl Kinases in Angiogenesis

Our studies have demonstrated that the Abl family kinases are activated following treatment with a variety of pro-angiogenic growth factors, including Angpt1, VEGF, and bFGF. These factors promote neovascularization by stimulating endothelial cell proliferation, survival, migration, and/or vascular stability [64,65] and thus represent potential therapeutic targets for the treatment of disorders involving aberrant, excessive angiogenesis. Interestingly, we observed that inhibition of Abl kinase activity impaired the ability of each of these growth factors to support endothelial cell survival. This finding raises the possibility that pharmacological targeting of the Abl kinases may have anti-angiogenic effects. Imatinib has shown anti-angiogenic activity in several tumor models [334,335,336], as well as inhibiting VEGF- and bFGF-driven angiogenesis *in vivo* [266]. While these anti-angiogenic effects have been attributed to imatinib-mediated inhibition of the PDGF receptor, an additional study demonstrated that expression of a dominant-negative Abl protein in endothelial cells results in reduced bFGF-driven angiogenesis *in vitro* and *in vivo*, as well as decreased tumor size and vascularization in an endothelial cell/breast cancer co-implantation xenograft tumor

model [337]. These findings suggest an *in vivo* pro-angiogenic function for the Abl family kinases.

In order to evaluate the potential angiogenic role of the endothelial Abl kinases, we examined subcutaneous xenograft tumor growth in endothelial *Abl* knockout mice, using both Lewis Lung Carcinoma (LLC) and B16-F10 melanoma syngeneic tumor models. Since Abl^{ECKO}; $Arg^{-/-}$ mice are embryonic lethal, these tumors were implanted into adult Abl^{ECKO}; $Arg^{+/-}$ mice, as well as $Arg^{+/-}$ control mice. However, no differences in LLC tumor growth (**Figure 5.3A**) or vascularization (**Figure 5.3B**) were observed in mice lacking endothelial *Abl* expression, although a trend toward decreased tumor growth was observed in the B16-F10 model (**Figure 5.3C,D**). It is likely that Arg can compensate for the effects of loss of endothelial *Abl* inactivation in these tumor models. Therefore, it may be useful to examine tumor vascularization using inducible endothelial *Abl* knockout mice (on an $Arg^{-/-}$ background), in order to bypass the lethality of constitutive loss of endothelial Abl/Arg expression. However, the normal overall vascular patterning, branching, and vessel density observed in Abl^{ECKO}; $Arg^{-/-}$ embryos suggest that the Abl kinases are not required for developmental angiogenesis. It remains to be determined whether endothelial Abl/Arg expression similarly is dispensable for pathological angiogenesis. Notably, treatment with the Abl/Arg-specific pharmacological inhibitor GNF-5 decreased growth of lung cancer xenograft tumors (Greuber, Ring, and Pendergast, unpublished data). While no obvious differences in

vascularization were observed in tumors from mice treated with GNF-5, these tumors displayed increased hypoxic area, suggesting that Abl kinase inhibition leads to defective function of the tumor vasculature. Further studies will be required to characterize the mechanisms by which GNF-5 exerts its anti-tumor effects.

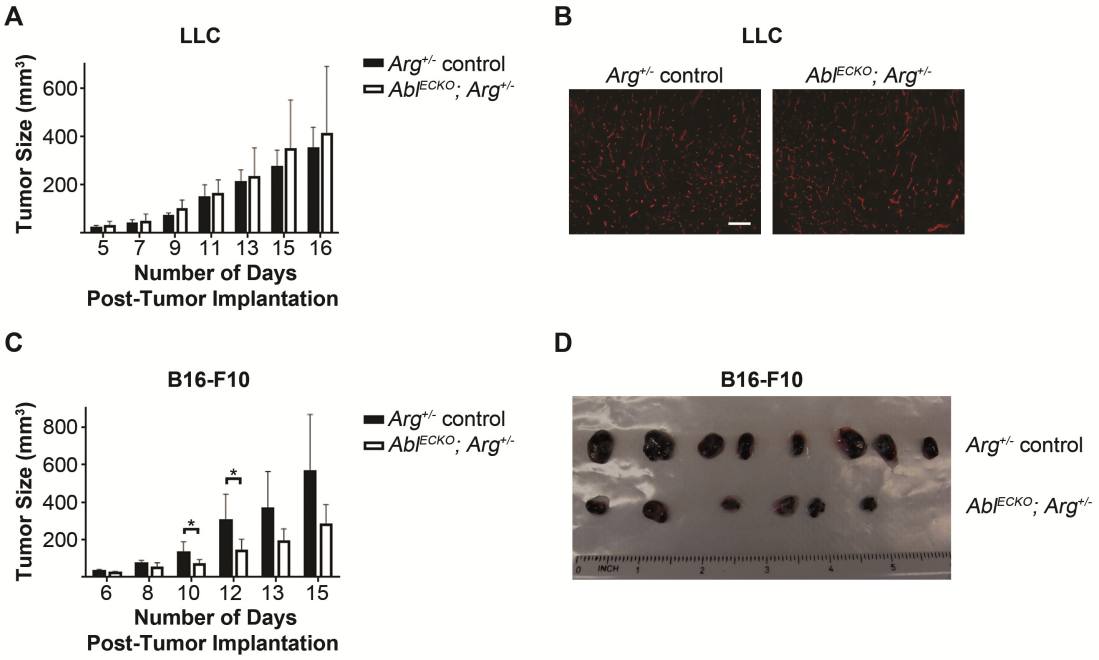

Figure 5.3 Xenograft Tumor Growth and Vascularization in *Abl^{ECKO}; Arg$^{+/-}$* Mice

Lewis lung carcinoma (LLC) or B16-F10 melanoma cells were implanted subcutaneously into separate cohorts of approximately 3-month-old *Abl^{ECKO}; Arg$^{+/-}$* mice and *Arg$^{+/-}$* controls ($5x10^5$ LLC cells; $1.5x10^5$ B16-F10 cells). Upon detection of palpable tumors, tumor dimensions were measured every 1-2 days using calipers and tumor volume calculated as volume = ½ * width^2 * length. (**A**) Assessment of growth of LLC tumors in *Abl^{ECKO}; Arg$^{+/-}$* mice and *Arg$^{+/-}$* controls. Data are presented as means +/- SD (n=8-11 mice per genotype). (**B**) CD31 immunostaining of LLC tumor cryosections from *Abl^{ECKO}; Arg$^{+/-}$* and *Arg$^{+/-}$* control mice, demonstrating no obvious differences in tumor vascularization. Scale bar = 100μm. (**C**) Assessment of growth of B16-F10 tumors in *Abl^{ECKO}; Arg$^{+/-}$* mice and *Arg$^{+/-}$* controls. Data are presented as means +/- SD (n=6-8 mice per genotype). (**D**) Image of B16-F10 tumors excised (15 days post- implantation) from the *Abl^{ECKO}; Arg$^{+/-}$*

and $Arg^{+/-}$ control mice in (**C**), showing a trend toward decreased tumor size in Abl^{ECKO}; $Arg^{+/-}$ mice. (*P<0.05).

5.4 Role of the Abl Kinases in Endothelial Permeability

5.4.1 Possible Implications for the Treatment of Pathological Vascular Permeability

In addition to its pro-angiogenic function, VEGF is a potent inducer of endothelial permeability [81], resulting in disruption of endothelial barrier function. While loosening of the endothelial barrier by VEGF and other soluble mediators is an important aspect of both normal angiogenic remodeling and inflammatory responses [5,30], abnormally elevated vascular permeability contributes to edema, elevated interstitial fluid pressure, and tissue damage in pathological conditions including cancer, sepsis, and ischemia-reperfusion injury [31,32]. Our studies have demonstrated that inhibition of the Abl family kinases using either imatinib or GNF-2 potently inhibits the induction of endothelial permeability by VEGF treatment both *in vitro* and *in vivo*. Importantly, reduced VEGF-induced vascular leakage also was observed in Abl^{ECKO}; $Arg^{+/-}$ mice. Abl kinase inhibition similarly decreased endothelial permeability in response to the inflammatory mediators thrombin and histamine. Together, these findings suggest that the Abl kinases play an important role in regulating endothelial barrier function and that pharmacological targeting of Abl/Arg thus may represent a potential treatment for disorders involving excessive vascular leakage.

Indeed, within the past year, two published studies documented beneficial effects of the Abl family kinase inhibitors imatinib or nilotinib in murine models of sepsis and LPS-induced acute lung injury (ALI) [344,345]. In these studies, imatinib or nilotinib treatment reduced vascular leakage and pulmonary edema. However, as both of these inhibitors target multiple kinases in addition to Abl/Arg [415], it remains unclear whether their protective effects are a result of inhibition of the Abl kinases or other targets. Imatinib and nilotinib also target multiple cell types in these *in vivo* disease models, raising the possibility that cell types other than endothelial cells may mediate the anti-permeability effects of these drugs. In this regard, imatinib and nilotinib treatment were found to decrease levels of pro-inflammatory cells (i.e., macrophages and neutrophils) and cytokines (TNFα, IL-6, IL-1β) in bronchoalveolar lavage fluid during LPS-induced ALI in mice [344]. As we observed decreased VEGF-induced permeability in mice lacking endothelial *Abl* expression (Abl^{ECKO}; $Arg^{+/-}$ mice), it may be of interest to determine whether vascular leakage induced by additional factors (histamine, thrombin, etc.) is similarly attenuated in these mice, as well as whether loss of the endothelial Abl kinase impacts vascular permeability and disease progression in mouse models of disorders involving excessive vascular leakage (including sepsis/ALI). A recent study suggested an important contribution of the Arg kinase to endothelial permeability responses *in vitro* [345]. Thus, endothelial *Abl* knockout alone may not be sufficient to impair permeability in disease models *in vivo*. An examination of the

specific endothelial role of the Arg kinase will require generation of conditional *Arg* knockout mice.

Seemingly in contrast to the endothelial barrier-enhancing effects of Abl kinase inhibition observed in these studies, peripheral edema is one of the most commonly reported side effects of imatinib treatment in chronic myelogenous leukemia patients [416]. However, use of the more potent Abl kinase inhibitor nilotinib has not been associated with similar clinical development of edema [270]. We typically did not observe altered baseline endothelial permeability upon Abl kinase inhibition, although an improvement in basal barrier function was observed in some experiments. However, the Abl kinases also have roles in signaling downstream of the endothelial barrier-promoting mediators S1P and Angpt1 [346,382]. Thus, further investigation will be needed to evaluate the effects of chronic Abl kinase inhibition on endothelial barrier function *in vivo*.

5.4.2 Anti-Permeability Mechanisms of Abl Kinase Inhibition

5.4.2.1 Adhesion and Contractility

Endothelial barrier function is governed in part by two opposing forces: the pro-adhesive effects of cell-cell junctions and cell-matrix adhesions and the barrier-destabilizing effects of acto-myosin contractility. Permeability-inducing factors upset this balance by disrupting endothelial cell-cell junctions [106,122,375,376,377], as well as generating increased cellular contractility [378,379]. We found that Abl kinase inhibition

in endothelial cells altered pro-adhesive signaling through activation of the Rac1 and Rap1 GTPases, which act to promote cortical actin remodeling and stabilize cell-cell junctions [190,191,192,384,385]. However, loss of Rac1 or Rap1 expression or function did not rescue the imatinib-mediated inhibition of VEGF-induced permeability, suggesting that the Abl kinases regulate endothelial barrier function through additional mechanisms. Interestingly, our studies demonstrated that loss of Abl kinase activity also decreases VEGF- and thrombin-induced generation of acto-myosin contractility, as assessed by phosphorylation of the myosin regulatory light chain (phospho-MLC2 S19). Thus, pharmacological targeting of the Abl kinases may impair endothelial permeability through multiple pathways, including strengthening cellular adhesions and inhibiting the barrier-disruptive force of acto-myosin contractility.

Previous work from our laboratory has shown a requirement for the Abl kinases in both formation and maintenance of epithelial adherens junctions [294]. Our current work suggests that Abl kinase activity is similarly required for the dissolution of endothelial cell-cell junctions in response to VEGF and inflammatory mediators. We observed that VEGF-induced activation of the Abl kinases is dependent in part upon the activity of Src family kinases, consistent with previous findings in PDGF-stimulated fibroblasts [279,286]. Src kinase activation plays an important role in VEGF-induced vascular permeability [102], through phosphorylation of the adherens junction protein VE-cadherin [103,104] , as well as regulation of VE-cadherin endocytosis and resulting

dissolution of endothelial cell-cell junctions [106]. However, we were unable to detect any changes in VE-cadherin phosphorylation upon VEGF stimulation, either in the absence or presence of Abl kinase inhibitors (data not shown). Intracellular association of VE-cadherin with β-catenin and α-catenin was also unchanged. While our inability to detect VEGF-induced VE-cadherin tyrosine phosphorylation may reflect technical limitations, due to the close association of the VE-cadherin complex with multiple protein tyrosine phosphatases, including vascular endothelial cell-specific phosphotyrosine phosphatase (VE-PTP) [417] and DEP-1 [50], this finding also suggests that additional mechanisms underlie the induction of endothelial permeability, independent of direct effects on the VE-cadherin protein complex.

In this regard, endothelial barrier-disrupting factors increase acto-myosin contractility and centripetal tension, which function to weaken intercellular adhesion, resulting in cellular retraction and formation of intercellular gaps [378,379]. Our finding that VEGF- or thrombin-induced phosphorylation of MLC2 (S19) was decreased following loss of Abl kinase function suggests that Abl kinase inhibition may impair endothelial permeability by attenuating the barrier-destabilizing effects of acto-myosin contractility. MLC2 (S19) phosphorylation is an important regulator of cellular contractility, as phosphorylation of this site promotes contractility by increasing myosin ATPase activity [98]. Previous work has linked the Abl kinases to regulation of acto-myosin contractility through phosphorylation of p190RhoGAP [321] . Abl/Arg-mediated

phosphorylation stimulates p190RhoGAP inhibitory activity [321], leading to reduced levels of active RhoA and decreased acto-myosin contractility [322,323]. Conversely, increased acto-myosin contractility, resulting in adherens junction destabilization, was observed following extended Abl kinase inhibition in epithelial cells [294]. In contrast, we did not observe any difference in thrombin-induced Rho activation in imatinib-treated endothelial cells, suggesting that the Abl kinases may play differential roles in the regulation of basal versus agonist-induced Rho activation and acto-myosin contractility in various cell types.

5.4.2.2 Calcium Signaling

Our observation that Abl kinase inhibition decreased agonist-induced MLC2 phosphorylation without affecting Rho GTPase activation suggests that loss of Abl kinase function may modulate activation of other pathways regulating acto-myosin contractility, such the Ca2+/calmodulin-dependent myosin light chain kinase (MLCK) [97]. Calcium signaling has a critical role in the regulation of endothelial permeability [386]. Endothelial cell stimulation with several permeability-inducing factors, including VEGF, thrombin, and histamine, leads to increased cytosolic Ca^{2+} levels [418,419], mediated by release of intracellular Ca^{2+} stores as well as extracellular Ca^{2+} entry through membrane channels [387,420]. Importantly, blocking this increase in cytosolic Ca^{2+} levels impairs induction of endothelial permeability [195,421,422,423,424]. In addition to promoting acto-myosin contractility through activation of MLCK, Ca^{2+} regulates the

activity of other enzymes important for endothelial permeability responses, including PKCα and eNOS [92,93,387,425,426].

Notably, we observed diminished Ca^{2+} mobilization in response to the permeability-inducing factors VEGF, thrombin, and histamine in the presence of the Abl kinase inhibitors imatinib or GNF-2. In the case of VEGF stimulation, this attenuated increase in cytosolic Ca^{2+} levels may result in part from delayed phosphorylation/ activation of PLCγ, which mediates release of Ca^{2+} from intracellular (ER) stores by catalyzing IP_3 production [387]. Interestingly, we also observed delayed phosphorylation of the VEGF receptor (VEGFR2) on tyrosine 1175, which is required for activation of PLCγ [82]. Further studies will be required to determine whether the Abl kinases can directly phosphorylate VEGFR2, as has been shown for the PDGF and EGF receptors [58,59], as well as whether phosphorylation of additional VEGFR2 tyrosine residues is affected by loss of Abl kinase activity. Interestingly, altered endosomal trafficking of VEGFR2 has been linked to protein tyrosine phosphatase (PTP)1b-mediated dephosphorylation of the Y1175 residue [427]. The Abl kinases have been implicated in EGF receptor endocytosis [392] and Notch receptor endocytic trafficking [413]. Thus, it is possible that the observed delay in VEGFR2 Y1175 phosphorylation may result from altered receptor localization/trafficking in the absence of Abl kinase activity.

While VEGF-mediated IP_3 production is mediated by VEGFR2-induced PLCγ activation, thrombin- and histamine-induced Ca^{2+} mobilization is regulated by G protein-mediated activation of PLCβ [387]. Thus, distinct mechanisms are likely to underlie the imatinib- and GNF-2-mediated inhibition of Ca^{2+} mobilization by these factors. Interestingly, Abl kinase inhibition also decreased Ca^{2+} mobilization in response to thapsigargin (**Figure 5.4A**), which inhibits sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) enzymes [428], resulting in increased cytosolic Ca^{2+} levels through passive depletion of ER stores. This finding suggests that the Abl kinases may regulate Ca^{2+} mobilization through IP_3-independent mechanisms. In this regard, previous studies have suggested a role for tyrosine kinases in regulating extracellular calcium entry through plasma membrane channels following depletion of intracellular Ca^{2+} stores (store-operated Ca^{2+} influx) [387].

To examine the relative effects of Abl kinase inhibition on release of intracellular Ca^{2+} stores versus extracellular Ca^{2+} entry, we evaluated VEGF-induced Ca^{2+} mobilization using a two-step protocol, in which VEGF stimulation is performed in the absence of extracellular Ca^{2+} (to examine release of intracellular Ca^{2+} stores), followed by the re-addition of extracellular Ca^{2+} (to examine extracellular Ca^{2+} entry). Interestingly, while VEGF-induced Ca^{2+} mobilization was markedly impaired by Abl kinase inhibition in the absence of extracellular Ca^{2+}, no consistent effect was observed on cytosolic Ca^{2+} levels following re-addition of extracellular Ca^{2+} (**Figure 5.4B** and data not shown).

These findings indicate that Abl kinase inhibition primarily impairs VEGF-induced release of Ca^{2+} from intracellular stores, rather than extracellular Ca^{2+} entry. Notably, thapsigargin-induced Ca^{2+} mobilization similarly was inhibited upon imatinib treatment in the absence of extracellular Ca^{2+} (**Figure 5.4C**), suggesting that the diminished VEGF-induced ER Ca^{2+} release upon Abl kinase inhibition may not simply result from decreased IP_3 generation.

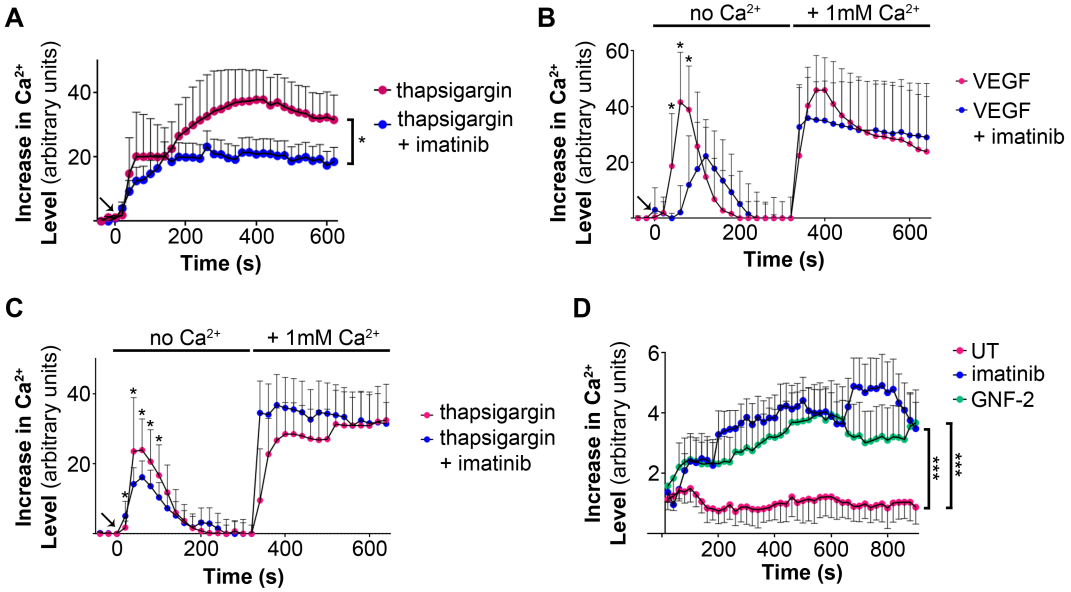

Figure 5.4 Altered Release of Intracellular Ca^{2+} Stores Following Abl Kinase Inhibition

(**A**) Quantification of intracellular Ca^{2+} levels in HMVECs stimulated with thapsigargin (1μM) with or without imatinib (10μM) pre-treatment. Values are expressed as increases in intracellular Ca^{2+} levels, relative to levels in unstimulated cells. Arrow indicates timing of thapsigargin treatment. Data are presented as means +/- SD of 35 cells per treatment and are representative of 3 independent experiments. (**B** and **C**) Analysis of intracellular Ca^{2+} levels in HMVECs treated with either (**B**) 100ng/mL VEGF or (**C**) 1μM thapsigargin, +/- imatinib (10μM) pre-treatment. Cells were stimulated with VEGF or thapsigargin in HBSS without Ca^{2+} for 5 minutes, followed by re-addition of 1mM extracellular Ca^{2+}.

Arrows indicate timing of VEGF/thapsigargin treatment. Values are expressed as increases in intracellular Ca^{2+} levels, relative to levels in unstimulated cells. Data are presented as means +/- SD of 35 cells per treatment and are representative of at least 3 independent experiments. (**D**) Assessment of intracellular Ca^{2+} levels in HMVECs treated with 10μM imatinib, 15μM GNF-2, or DMSO vehicle control (UT) for 15 minutes. Values are expressed as increases in intracellular Ca^{2+} levels, relative to levels in unstimulated cells. Data are shown as means +/- SD (35 cells per treatment) and are representative of 3 independent experiments. (*P<0.05; ***P<0.001).

This finding further raises the possibility that Abl kinase inhibition may alter the overall content of the ER Ca^{2+} stores. In this regard, transient inhibition of the Abl kinases with either imatinib or GNF-2 rapidly (within 15 minutes) led to mildly increased levels of intracellular Ca^{2+} (**Figure 5.4D**). It remains to be determined if this increase in cytosolic Ca^{2+} levels results from stimulation of extracellular Ca^{2+} entry or, perhaps more intriguingly, from Ca^{2+} leakage from intracellular stores. It also would be important to demonstrate that these effects on Ca^{2+} levels are a result of Abl/Arg kinase inhibition (for instance, through expression of imatinib-resistant Abl and Arg proteins and/or Abl/Arg depletion), rather than off-target effects of the pharmacological inhibitors. Thapsigargin-induced depletion of ER Ca^{2+} stores has been linked to induction of ER stress [429,430]. Imatinib treatment leads to an ER stress response in cardiomyocytes [350], as well as in BCR-ABL1-expressing 32Dcl3 myeloid precursor cells [431], which contributes to increased cell death. Thus, it is tempting to speculate that this imatinib-induced ER stress may result from altered ER calcium homeostasis; however, further experimentation will be required to test this possibility.

Regardless of the mechanism whereby Abl kinase inhibition alters agonist-induced Ca^{2+} mobilization, it also will be important to examine whether the observed decrease in cytosolic Ca^{2+} levels inhibits activation of Ca^{2+}-regulated enzymes involved in endothelial barrier dysfunction, such as MLCK and PKCα [387]. Notably, imatinib did not inhibit endothelial permeability induced by the PKC activator PMA (phorbol 12-myristate 13-acetate) (**Figure 5.5**), suggesting that PKC may function downstream of Abl in pathways regulating endothelial permeability. Alternatively, the induction of permeability by PMA may occur through Abl-independent pathways distinct from those activated by VEGF and inflammatory mediators.

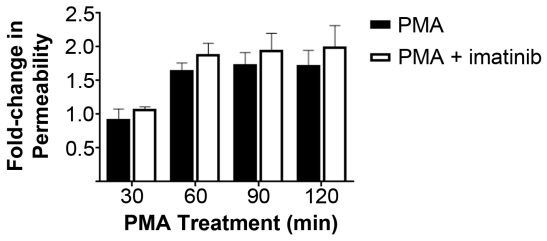

Figure 5.5 No Effect of Abl Kinase Inhibition on Endothelial Permeability Induced by PMA Treatment

Evaluation of permeability of HMVECs to fluorescein-labeled dextran following treatment with PMA (100nM) for the indicated times, +/- 10μM imatinib. Data are shown as fold-changes in permeability, relative to unstimulated cells, +/- SEM (n=3).

In addition to affecting cellular Ca^{2+} levels, Abl may regulate MLCK activation through phosphorylation. Abl-mediated phosphorylation of MLCK (Y464) has been observed following S1P treatment of endothelial cells [346]; tyrosine phosphorylation of

MLCK has been linked to enhanced enzymatic activity at lower Ca^{2+} concentrations [391]. It remains to be determined whether Abl kinase activation by endothelial permeability-inducing mediators similarly may contribute to MLCK phosphorylation and activation. It is possible that decreased intracellular Ca^{2+} levels in cells with reduced Abl kinase activity might contribute to impaired agonist-stimulated MLCK activity, leading to the observed reduction in MLC2 phosphorylation. Further investigation is needed to characterize fully the effects of Abl kinase inhibition on MLCK activity, along with other Ca^{2+}-regulated mediators of endothelial permeability.

5.5 Implications for Clinical Use of Abl Kinase Inhibitors?

In all, our work has demonstrated important roles for the Abl kinases in the regulation of endothelial cell function both *in vitro* and *in vivo*, including growth factor-mediated survival responses as well as induction of endothelial permeability by a diverse group of agonists. As previously discussed, our finding that Abl kinase inhibition impaired endothelial barrier dysfunction suggests that pharmacological targeting of the Abl kinases may have beneficial effects in treatment of disorders involving pathological vascular leakage. In addition to decreasing pulmonary edema in murine models of sepsis/ALI [344,345], Abl kinase inhibitors could potentially protect against VEGF-induced edema and tissue damage following infarction and/or ameliorate the increased vessel leakiness and elevated interstitial fluid pressure observed in tumors [32]. In this regard, imatinib treatment was previously shown to decrease interstitial

fluid pressure in lung and colon cancer models, resulting in improved tumor oxygenation and drug delivery [340,341,342].

In contrast, we found that loss of endothelial expression of the Abl family kinases resulted in late-stage embryonic and perinatal lethality in mice, as well as localized vascular loss and tissue damage, suggesting deleterious vascular effects of loss of Abl kinase function. However, it is important to note several distinctions between the loss of Abl function in the mouse model of endothelial *Abl* depletion and the clinical use of Abl kinase inhibitors. First, in our knockout mice, endothelial cells lacked Abl kinase expression from the onset of vascular development. Thus, the phenotypes observed in Abl^{ECKO}; $Arg^{-/-}$ embryos and Abl^{ECKO}; $Arg^{+/-}$ adult mice may result from abnormalities in embryonic vascular development and/or effects of chronic loss of Abl kinase function. Examining the effects of loss of endothelial Abl kinase expression in adulthood, which would be more representative of the context in which Abl kinase inhibitors are used clinically, would require the generation of an inducible endothelial *Abl* knockout model (i.e, *VE-cadherin-CreER*^{T2}, *Tie2-CreER*^{T2} or *Pdgfb-iCreER*-mediated *Abl* inactivation [394,395,396,397]). Second, endothelial *Abl* depletion results in complete absence of Abl protein expression, which leads to loss of the scaffolding functions of the Abl kinase in addition to loss of its kinase activity. Endothelial-specific knock-in of a kinase-inactive mutant *Abl* allele (Abl-K290R, [432]) could allow for the assessment of the role of Abl tyrosine kinase activity in endothelial function. Alternatively, generation of tissue-

specific knock-in mice expressing an imatinib-resistant Abl kinase (Abl-T315I mutation, [414]) would enable the assessment of the contribution of Abl kinase inhibition in endothelial cells (or cardiomyocytes) to the cardiovascular complications of imatinib therapy.

However, despite reports of adverse cardiovascular effects in a subset of imatinib-treated patients [350,351,352], incidence of these complications appears to be low, and imatinib is generally well tolerated [368]. Similarly, overt cardiovascular phenotypes were observed only in a subset of adult Abl^{ECKO}; $Arg^{+/-}$ mice, suggesting that additional factors (genetic and/or environmental) are required for development of pathological cardiovascular phenotypes. Accordingly, genetic and environmental differences in patients are likely to underlie the observed clinical variability in cardiovascular complications of imatinib. Thus, it would be of interest to determine how genetic differences may modify the effects of endothelial Abl loss-of-function in our mouse model, as well as whether loss of endothelial Abl kinase function may modify the progression of cardiovascular pathologies such as diabetes and atherosclerosis. These studies could be particularly relevant, given that the requirement for chronic use of Abl kinase inhibitors likely will increase the proportion of patients treated with these drugs in the context of cardiovascular co-morbidities.

In summary, the current studies have demonstrated a requirement for endothelial expression of the Abl family kinases in vascular development and function

in vivo, as well as in mediating endothelial cell survival and permeability responses to a variety of soluble mediators. Further studies will be needed to examine the potential utility of pharmacological targeting of the Abl kinases for treatment of disorders involving deregulated vascular permeability and angiogenesis, as well as to understand the precise mechanisms underlying the adverse effects of depletion of the endothelial Abl kinases on *in vivo* vascular function. An improved understanding of these contrasting beneficial and deleterious effects of loss of endothelial Abl kinase function will be useful for optimizing the clinical use of Abl kinase inhibitors, in order to reduce potential cardiovascular complications.

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Biography

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EDUCATION

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PUBLICATIONS

- **Chislock, EM**, Pendergast AM (2013) Abl family kinases regulate endothelial barrier function *in vitro* and in mice. *PLoS ONE*, revision submitted.
- **Chislock, EM**, Pendergast AM (2013) Tie2 (to) Abl: signaling to endothelial cell survival. *Cell Cycle* 12(24). <http://dx.doi.org/10.4161/cc.26877>
- **Chislock EM**, Ring C, Pendergast AM (2013) Abl kinases are required for vascular function, Tie2 expression, and angiopoietin-1-mediated survival. *Proc Natl Acad Sci U S A* 110(30):12432-12437.
- Gordon KJ, Dong M, **Chislock EM**, Fields TA, Blobe GC (2008) Loss of type III transforming growth factor beta receptor expression increases motility and invasiveness associated with epithelial to mesenchymal transition during pancreatic cancer progression. *Carcinogenesis* 29(2):252-262.
- Kinder M, **Chislock E**, Bussard KM, Shuman L, Mastro AM (2008) Metastatic breast cancer induces an osteoblast inflammatory response. *Exp Cell Res* 314(1):173-183.
- Zipfel PA, Bunnell SC, Witherow DS, Gu JJ, **Chislock EM**, Ring C, Pendergast AM (2006) Role for the Abi/wave protein complex in T cell receptor-mediated proliferation and cytoskeletal remodeling. *Curr Biol* 16(1):35-46.
- Mastro AM, Gay CV, Welch DR, Donahue HJ, Jewell J, Mercer R, DiGirolamo D, **Chislock EM**, Guttridge K (2004) Breast cancer cells induce osteoblast apoptosis: a possible contributor to bone degradation. *J Cell Biochem* 91(2):265-276.

HONORS & AWARDS

- James B. Duke Fellowship, Duke University, 2005
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- Phi Beta Kappa, Lambda Chapter of the Pennsylvania State University, 2006
- Duke Cancer Center Young Investigator, 2008
- American Heart Association Pre-Doctoral Fellowship (Mid-Atlantic Affiliate), 2008-2010
- PhRMA Foundation Pre-Doctoral Fellowship in Pharmacology/Toxicology, 2011-2012
- Robert J. Fitzgerald Scholar Award, Duke University Department of Pharmacology and Cancer Biology, 2013